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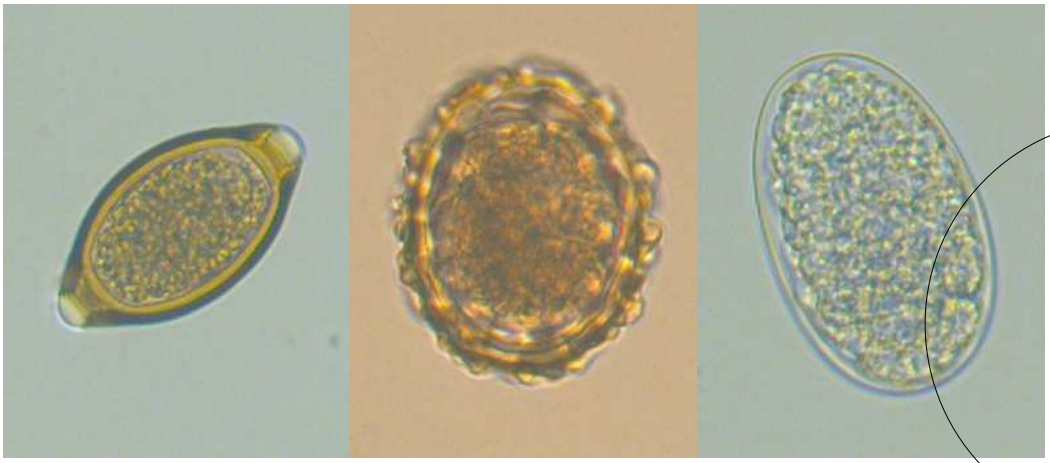
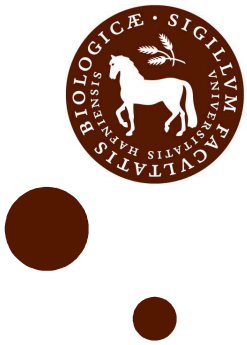


DEPARTMENT OF VETERINARY DISEASE BIOLOGY
FACULTY OF LIFE SCIENCE, UNIVERSITY OF COPENHAGEN

Sedimentation and resuspension of helminth eggs in water

Mita Eva Sengupta

PhD thesis



Copenhagen, January 2012

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Title of PhD thesis: Sedimentation and resuspension of helminth eggs in water

Subject area: Environmental hygiene; Parasitology.

Phd thesis by:

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Submitted: January 2012

Preface

The work presented in my PhD thesis has been performed from October 2008 to January 2012 at the Department of Veterinary Disease Biology, Section for Parasitology, Health, and Development, LIFE, and at the Department of Geography and Geology, both at the University of Copenhagen. Anders Dalsgaard from the section for Microbiology was my main supervisor, and Stig M. Thamsborg and Annette Olsen from the section for Parasitology, Health, and Development were my project supervisor and co-supervisor, respectively. At the Department of Geography and Geology I received technical supervision from Thorbjørn Joest Andersen. I did collaborative research with Bernard Keraita from Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, and collaborated with the Copenhagen wastewater treatment plant "Lynetten".

My thesis is based on the three papers listed below:

I. **Sengupta, M. E.,** Thamsborg, S. T., Andersen, T. J., Olsen, A., and Dalsgaard, A. 2011. Sedimentation of helminth eggs in water. Water Research 45:4651-4660.

II. **Sengupta, M. E.,** Andersen, T. J., Dalsgaard, A., Olsen, A. and Thamsborg, S. M.. Resuspension and settling of helminth eggs in water: interactions with cohesive sediments. Submitted to Water Research.

III. **Sengupta, M. E.,** Keraita, B., Olsen, A., Boateng, O. K., Thamsborg, S. M., Pálsdóttir, G. P, and Dalsgaard, A. Use of *Moringa oleifera* seed extracts to reduce helminth eggs and turbidity in irrigation water. Submitted to Water Research.

Mita Eva Sengupta
Copenhagen, January 2012

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Summary

Wastewater use in agriculture has a long history and it has been estimated that about 20 million hectares of agricultural land worldwide is irrigated with treated or untreated wastewater. The major health risks from irrigation with wastewater are associated with viral, bacterial, and parasite pathogens that are usually present in untreated, partially treated, and occasionally also in treated wastewater. Helminth parasite (*Ascaris*, *Trichuris*, and hookworm) eggs in wastewater are of particular health concern due to the high burden of helminthic diseases in low-income and lower-middle-income countries where use of wastewater is most prominent. In both conventional wastewater treatment and in natural waters (including faecal contaminated water) coagulation-flocculation and sedimentation are some of the fundamental physical processes that remove particles and helminth eggs from the water. However knowledge of how these processes work on helminth eggs and how the eggs behave in different types of water is very limited. To address this lack of knowledge three sub-studies were undertaken; two sub-studies to 1) determine the settling velocity and resuspension (erosion) rate of different types of helminth eggs, and one sub-study to 2) investigate under laboratory and field conditions to what extent seed extracts of *Moringa oleifera* can be used as a coagulant to increase the settling velocity of helminth eggs in water.

Sub-study I determined the settling velocity of helminth eggs in tap water and their interaction with suspended wastewater particles. The settling velocity of eggs in tap water, measured by settling of eggs in Owen tubes, was compared to theoretical settling velocities calculated by Stoke's law. The mean settling velocity in tap water of 0.061 mm s^{-1} found for *Ascaris suum* eggs was significantly lower than the corresponding values of 0.149 mm s^{-1} for *Trichuris suis* and 0.126 mm s^{-1} for *Oesophagostomum* spp. eggs, with the latter used as an indicator of hookworm eggs. For *T. suis* and *Oesophagostomum* spp. eggs the theoretical settling velocities were comparable with the observed velocities while it was three times higher for *A. suum* eggs. In wastewater, the mean settling velocity for *A. suum* eggs (0.158 mm s^{-1}) was found to be different from that of *T. suis* (0.087 mm s^{-1}), *Oesophagostomum* spp. (0.105 mm s^{-1}), and wastewater particles (0.047 mm s^{-1}). This indicates that in wastewater the eggs are incorporated into particle flocs with different settling velocities and that the settling velocity of eggs and particles is closely associated.

Sub-study II investigated resuspension of helminth eggs after sedimentation and interaction with cohesive sediment and suspended particles. The erodibility of both sediment and helminth eggs was

found to decrease over time indicating that sediment consolidation takes place along with incorporation of eggs into the sediment. This was supported by the fact that a higher settling velocity was found for eggs associated with particles as compared to eggs in clean water, which confirms the findings from **sub-study I**. The incorporation into the sediment bed decreases the mobility of helminth eggs and the aggregation increases the effective settling velocity of the eggs also leading to reduced mobility. Recalculation of the erosion threshold for helminth eggs and sediment showed that even at relatively low water velocities, i.e. 0.07-0.12 m s^{-1}, the eggs are likely to demonstrate high mobility in open irrigation channels. Our findings document that helminth eggs should not be viewed as single entities in water systems when modelling the distribution of eggs since both settling velocity and erodibility of eggs are determined by the mobility of particles and sediment present in the water stream.

Sub-study III explored to which extent the settling velocity of helminth eggs could be enhanced by the presence of seed extracts of *Moringa oleifera* (MO), thereby introducing a low technology and locally adapted water treatment method for helminth egg removal in low quality water to be used for crop irrigation. In medium to high turbid water MO seed extracts were effective in reducing the number of helminth eggs by 94-99.5% to 1-2 eggs per litre and the turbidity to 7-11 NTU which is an 85-96% reduction. MO is readily available in many tropical countries and can be used by farmers to treat highly turbid water for irrigation. However, additional improvements of water quality, e.g. by sand filtration, is suggested to meet the guideline value of ≤ 1 helminth egg per litre and a turbidity of ≤ 5 NTU as recommended by the World Health Organization. A positive correlation was established between reduction in turbidity and concentration of helminth eggs in irrigation water, turbid water, and wastewater treated with MO. The results indicate that MO facilitates the attachment of helminth eggs to suspended particles and/or flocs in the water, and that turbidity and concentration of helminth eggs are then reduced with the settling flocs.

The knowledge obtained on helminth eggs in water and their interaction with particles should be used when evaluating existing water treatment processes and enumeration methods of helminth eggs to ensure safe irrigation water. Additionally the knowledge could also be used to develop and enhance models for predicting the fate of helminth eggs in natural settings.

Sammendrag (Danish summary)

Brugen af spildevand i landbruget går langt tilbage i historien, og det anslås at omkring 20 millioner hektar landbrugsjord globalt set vandes med renset eller urenset spildevand. Den største sundhedsrisiko ved vanding med spildevand består i virale, bakterielle og parasitære patogener, som oftest forefindes i urenset og delvist renset spildevand, og somme tider også i renset spildevand. Æg fra helminth-parasitter (*Ascaris*, *Trichuris*, og hageorm) og de deraf følgende sygdomme udgør et særlig stort problem i fattige lande, hvor brugen af spildevand er mest udbredt. I både konventionel spildevandsrensning og i naturlige vandmiljøer (inkl. områder forurenet med fækalier) er koagulering/flokkulering og sedimentering nogle af de grundlæggende fysiske processer som anvendes til at fjerne partikler og helminth-æg fra vandet. Imidlertid er der kun et meget begrænset kendskab til, hvordan disse processer virker for helminth-æg, og hvordan æggene opfører sig i forskellige vandtyper. Med henblik på at øge vores viden på dette område blev følgende tre delstudier udført; to delstudier med det formål 1) at bestemme faldhastighed og erosionsrate for forskellige typer af helminth-æg, og et delstudie med det formål 2) at undersøge i hvor høj grad frøekstrakt fra planten *Moringa oleifera* kan anvendes som koaguleringsmiddel til at øge faldhastigheden af helminth-æg i vand.

I **delstudie I** bestemmes faldhastigheden for helminth-æg i rent vand (Københavns drikkevand), samt deres vekselvirkning med spildevandspartikler. Den målte faldhastighed for æg i rent vand, baseret på sedimentationsforsøg med æg i Owen-rør, sammenholdes med teoretiske værdier beregnet ved hjælp af Stokes' lov. Den gennemsnitlige faldhastighed for æg af typen *A. suum* måles til 0,061 mm s^{-1}, en betydelig lavere værdi end de tilsvarende på 0,149 mm s^{-1} og 0,126 mm s^{-1} for ægtyperne *Trichuris suis* hhv. *Oesophagostomum* spp. Sidstnævnte bruges som indikator for hageormsæg. For *T. suis* og *Oesophagostomum* spp. er den teoretiske faldhastighed sammenlignelig med de målte værdier, mens den for ægtypen *A. suum* er tre gange højere. I spildevand måles den gennemsnitlige faldhastighed for *A. suum* til 0,158 mm s^{-1}, hvilket er markant forskelligt for de tilsvarende tal for *T. suis* (0,087 mm s^{-1}), *Oesophagostomum* spp. (0,105 mm s^{-1}) og spildevandspartikler (0,047 mm s^{-1}). Disse resultater tyder på, at æggene i spildevand indkorporeres i partikel-flokke med forskellige faldhastigheder, og at der dermed er en tæt sammenhæng mellem faldhastighed af æg og partikler.

I **delstudie II** undersøges resuspension af helminth-æg efter sedimentering, og deres vekselvirkning med mudder sediment og svævende partikler i vandet. Det påvises at eosionsgraden for både sediment og helminth-æg aftager med tiden, hvilket indikerer en konsolidering af sedimentet samtidigt med indkorporering af æggene i sedimentet. Denne antagelse støttes af det faktum, at der påvises en højere faldhastighed for æg sammen med partikler sammenlignet med æg i rent vand, hvilket desuden bekræfter resultaterne fra **delstudie I**. Indkorporeringen af æg i sedimentlaget nedsætter deres mobilitet, og det samme gør den øgede effektive faldhastighed, som skyldes sammenklæbning mellem æg og partikler. Beregning af tærskelværdier for erosion af helminth-æg og sediment viser, at selv ved lave vandhastigheder på 0,07 – 0,12 m s^{-1} vil æggene sandsynligvis udvise høj mobilitet i åbne vandingskanaler. Helminth-æg ikke skal betragtes som selvstændige enheder ved modellering af deres fordeling i virkelige vandsystemer, eftersom både faldhastighed og erosionsgraden også bestemmes af mobiliteten af tilstedeværende partikler og sediment.

Delstudie III undersøger i hvor høj grad faldhastigheden for helminth-æg kan øges ved tilsætning af frøekstrakt fra *Moringa oleifera* (MO), og dermed introduceres en lavteknologisk rensningsmetode for reduktion af helminth-æg i vand af ringe kvalitet. I vandtyper med middel til høj turbiditet påvises det, at MO frøekstrakt effektivt reducerer antallet af helminth-æg med 94-99,5% til 1-2 æg per liter, og turbiditeten til 7-11 NTU svarende til en reduktion på 85-96%. MO er en let tilgængelig ressource i mange tropiske lande og kan anvendes af landmændene selv til at opnå en vis rensning af vand med høj turbiditet. Imidlertid kræves yderligere rensning med f.eks. sandfiltre for at sikre overholdelse af WHO's anbefaling om $\leq$ 1 helminth-æg per liter og en turbiditet $\leq$ 5 NTU. Delstudiet påviser yderligere en positiv korrelation mellem faldet i turbiditet og koncentrationen af helminth-æg i vandingsvand, turbidt vand og spildevand behandlet med MO. Resultaterne indikerer, at MO medvirker til at helminth-æg binder sig til svævende partikler og/eller flokke i vandet, og at turbiditet og koncentrationen af helminth-æg efterfølgende reduceres ved bundfældning disse flokke.

Den opnåede viden om helminth-æggenes egenskaber i vand og deres vekselvirkning med spildevandspartikler vil kunne anvendes til evaluering af eksisterende vandrensningsprocesser og æg kvantificeringsmetoder, med det formål at sikre rent vandingsvand. Endvidere kan resultaterne bruges til at udvikle og forbedre modeller til beregning af helminth-æggenes opførsel og skæbne i naturlige vandsystemer.

Chapter 1

Introduction

Population growth in water scarce areas and pressure on the world's water resources is responsible for an increased competition between urban and agricultural need for freshwater. In low-income countries, wastewater is often the only source of water available for irrigation due to lack of alternatives and proper treatment facilities. These countries do not have the capacity to properly treat wastewater, so it is discharged into water bodies which are then used for irrigation with no or little treatment (WHO and UNICEF, 2000).

In this thesis the term 'wastewater' refers to the liquid part of waste from households (black and grey water), farms, and industrial establishments, and it is often mixed with groundwater, surface water, and storm water (Metcalf and Eddy, 1995). Wastewater use in agriculture has a long history and today millions of farmers globally use wastewater for agricultural production. It has been estimated that about 20 million hectares of agricultural land worldwide is irrigated with treated or untreated wastewater (Jimenez and Asano, 2008) and at least 10% of the world's population consume food produced by irrigation with wastewater (Smit and Nasr, 1992).

In many low-income countries poor transportation and limited availability of cold storage facilities makes it necessary to grow crops close to the cities and the consumers. The available water sources for urban or peri-urban agriculture are wastewater or other types of low quality water. In many African cities 50% to 90% of consumed vegetables are produced within or close to the city using polluted irrigation water (Drechel *et al.*, 2006). In Pakistan, at least 26% of the country's vegetables are irrigated with wastewater and any reduction in this practice would reduce food supply to the cities (Ensink *et al.*, 2004). In Hanoi, Vietnam, 80% of vegetable production is practiced in urban and peri-urban areas irrigated with wastewater and water from the Red River Delta, which receives drainage effluent from the city itself (Lai, 2002).

When untreated wastewater is used for crop irrigation it poses substantial risks to human health, not only for farmers, but also for surrounding communities and consumers of the crops, in particular when crops are eaten uncooked (Blumenthal *et al.*, 2000). The major health risks are associated with excreta-related pathogens which are usually present in untreated and partially treated wastewater (Feachem *et al.*, 1983; Shuval *et al.*, 1986). Excreta-related pathogens most commonly found in wastewater are faecal bacteria (*Campylobacter* spp., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio cholera*), helminths (roundworm, whipworm, and hookworms), protozoa (*Entamoeba histolytica*, *Cryptosporidium parvum*, and *Giardia intestinalis*), and viruses (enteric viruses, hepatitis A virus, and rotaviruses). Main health problems associated with these pathogens are diarrhoeal disease and helminth infections, which have a high morbidity in low-income countries (Feachem *et al.*, 1983).

Helminth parasite eggs in wastewater are of particular health concern due to the high burden of helminthic diseases in low-income and lower-middle-income countries where use of wastewater is most prominent. The most important intestinal worms are *Ascaris lumbricoides* (human roundworm), *Trichuris trichiura* (human whipworm), and *Ancylostoma duodenale* and *Necator americanus* (human hookworms). It is estimated that at least 1.2 billion people globally are infected with one or more of these intestinal parasites (Bethony *et al.*, 2006; de Silva *et al.*, 2003). Helminth eggs can survive in the environment for several years and stay infective, especially in wastewater which offers protection against desiccation and direct sunlight. In its recent guidelines the World Health Organization (WHO) recommends that wastewater used for irrigation should contain less than one helminth egg per litre when no other effective risk reduction options are available (WHO, 2006).

In both conventional wastewater treatment (Fig. 1.1) and in natural waters coagulation-flocculation and sedimentation are some of the fundamental physical processes which can help reduce water pollution (Elimelech *et al.*, 1995). However, very little information exists on how helminth eggs behave in water both with regard to sedimentation and flocculation. Until now

Fig. 1.1 Settling tank at the conventional wastewater treatment plant Lynetten in Copenhagen, Denmark. http://lynettefaellesskabetis.net.dynamicweb.dk

only theoretical calculations of settling velocities for helminth eggs in clean water have been proposed by Shuval (1978). No data is available on the incorporation of deposited helminth eggs into the sediment and their possible resuspension which can lead to an increased concentration of eggs in the water. In wastewater, some adhesion of helminth eggs to particles is expected, but it remains unknown to what extent this occurs.

The settling velocity of helminth eggs is a determinant factor for the sedimentation process occurring in water with little or no turbulence. In flowing water, on the other hand, the resuspension rate of settled eggs becomes an equally important factor in relation to transport and mobility. Flocculation in its turn is known to modify the settling velocity of particles (Droppo and Ongley, 1994) and thus, ideally, the combined effects of settling, resuspension, and flocculation should be taken into account when predicting or modelling helminth egg behaviour in different types of water used for irrigation of food crops. Flocculation in itself offers a means to effectively control sedimentation through its effect on the settling velocity. Conventional wastewater treatment typically involves the use of chemical coagulants to enhance coagulation-flocculation and hence sedimentation, but some biological materials such as extracts of *Moringa oleifera* seeds are known to act as natural particle coagulants (Jahn, 1988). Due to their low cost and wide availability the possible use of biological agents for helminth egg removal would be particularly interesting for low-income countries, but today it remains unknown whether seed extracts can be used for this purpose. A better understanding of helminth egg behaviour in water based on above effects could potentially help to improve irrigation water quality and thus reduce health risks for millions of people around the world.

1.1 Objectives and thesis organization

In the present PhD thesis the overall objective is to increase our understanding of how helminth eggs behave in different types of water, ranging from freshwater to wastewater.

The specific objectives are:

- To determine the settling velocity and resuspension rate of different types of helminth eggs.
- To investigate under laboratory and field conditions to what extent seed extracts of *Moringa oleifera* can be used as a coagulant to increase settling velocity of helminth eggs in water.

The thesis is presented in seven chapters. **Chapter 1** provides an introduction to the overall context of the study along with the rationale and objectives. **Chapter 2** presents an in-depth background to helminth eggs and particles, and their adhesion, flocculation, settling, and resuspension as well as other subject areas covered by the thesis. The general methods used are summarized in **Chapter 3**. To address the objectives, the study was organized into three sub-studies and the results and discussions of these are presented as three scientific papers in **Chapter 4** along with some additional results. In **Chapter 5** an overall discussion of the main results and their implications are presented as well as perspectives. **Chapter 6** comprises conclusions and future studies, and **Chapter 7** is a list of references used in the thesis.

Chapter 2

Background

2.1 Helminth eggs and their ecology

The human helminth species, *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (*Ancylostoma duodenale* and *Necator americanus*) are different from each other with respect to life cycle and epidemiology. However, all species have a direct lifecycle where no intermediate host is needed (Bethony *et al.*, 2006; de Silva *et al.*, 2003). Eggs are shed by infected humans in whom the female worms produce eggs and hence these people serve as the source of helminth eggs into faecal contaminated water (Shuval *et al.*, 1986). They cannot however multiply outside the final host and the eggs require a period of development in the environment before they become infective eggs or free-living larvae (Bethony *et al.*, 2006; de Silva *et al.*, 2003).

The pig helminths, *Ascaris suum*, *Trichuris suis*, and *Oesophagostomum* spp. (Fig. 2.1) are often used as model organisms for the corresponding human helminths (Boes and Helwigh, 2000), i.e. *A. lumbricoides*, *T. trichiura*, and hookworm, respectively. The survival rate in the environment of pig helminth eggs, *A. suum* and *T. suis*, and of corresponding human helminth eggs is more or less the same, and egg morphology and size is also virtually identical (Beer, 1976; Alicata, 1935). Discussions are ongoing whether *Ascaris* from pigs and from humans as well as *Trichuris* from pigs and from humans are separate species, respectively (Nejsum *et al.*, 2012). The three pig helminth

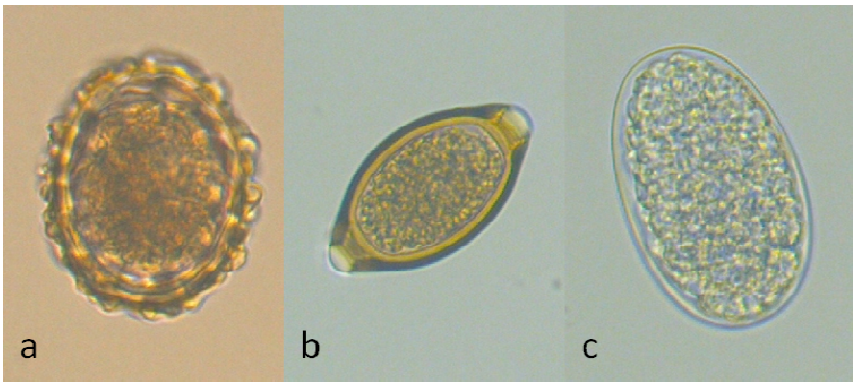

Fig. 2.1 Morphology of the three pig helminth eggs. Not to scale. Photos by the author.
a. *Ascaris suum*;
b. *Trichuris suis*;
c. *Oesophagostomum* spp.

eggs are relatively easy to obtain in high numbers from infected pigs in Denmark and have been used throughout this study. Note that throughout this thesis the term 'helminth' refers to nematodes only unless stated otherwise.

In the following subsections a description of the helminth egg morphology (section 2.1.1), survival (section 2.1.2), and adhesive properties (section 2.1.3) will be presented. These are central for understanding the behaviour and fate of helminth eggs in an aqueous environment, such as wastewater.

2.1.1 The helminth egg shell

The morphology of the three helminth egg types is very different (see Fig. 2.1). While the characteristic appearance of *Ascaris* and *Trichuris* egg types makes them easily recognized, the *Oesophagostomum* egg type does not appear to differ much from eggs of other strongyles and free-living nematodes (Wharton, 1980). The *Ascaris* egg type is round to elliptical in shape and the surface is irregularly mammillated, *Trichuris* egg types are lemon shaped with a knob at each pole (polar plugs) and have a smooth surface whereas the egg type of *Oesophagostomum* egg type is elliptical with a smooth surface (Alicata, 1935; Beer, 1973b; Ubelaker and Allison, 1975). The *Ascaris* and *Trichuris* egg shell is thick and consists of three layers (Fig. 2.2) formed by the egg (Wharton, 1980; Foor, 1967). The inner thin lipid layer is responsible for the high level of impermeability; the thick middle chitinous layer provides structural strength that is further increased by a system of interconnecting ridges; and the outer thin vitelline layer derives from the vitelline membrane of the oocyte. Furthermore, *Ascaris* eggs have an outermost uterine-derived layer consisting of a proteinaceous material which is responsible for the mammilated appearance of the egg. In the passage through the intestine the colorless uterine layer becomes stabilized by a quinine-tanning process making the egg shell brown and chemically inert (Wharton, 1980). The *Oesophagostomum* egg shell also consists of the three layers mentioned, however each layer being thinner resulting in a more fragile egg shell than the other two egg types (Alicata, 1935). The differences seen in egg shells are related to the life cycle of the parasite where *Ascaris* and *Trichuris* egg types in

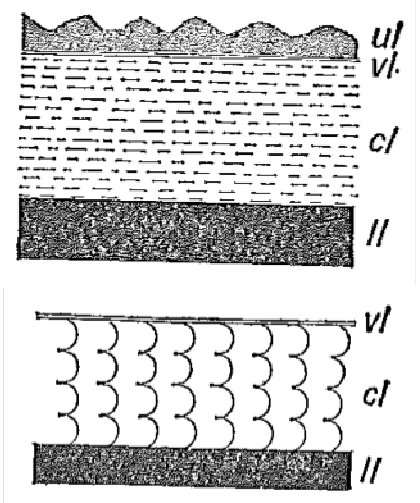

Fig 2.2 The egg shell structure of *Ascaris lumbricoides* (above) and *Trichuis suis* (below). Layers: *ul* –uterine, *vl* –vitelline, *cl* –chitinous, and *ll* –lipid layer. (Wharton, 1980).

the environment develop into an infective egg containing a larva, whereas *Oesophagostomum* egg types hatch L_1-larvae which develop into infective L_3-larvae (Fig. 2.3).

Fig. 2.3 Life cycle of *A. suum* (outer cycle), *Oesophagostomum* spp. (middle cycle), and *T. suis* (inner cycle) (Roepstorff and Nansen, 1998).

2.1.2 Development, resistance, and survival of helminth eggs

The eggs of *A. suum* and *T. suis* are generally considered to survive for many years and remain infective in soil for 6-15 years (Müller, 1953; Krasnos, 1978) and at least 6-11 years (Burden *et al.*, 1987; Hill, 1957), respectively. However studies have shown that the majority of eggs died within one month after deposition onto pastures with only a small proportion of the eggs surviving for a year or more (Larsen and Roepstorff, 1999; Burden and Hammet, 1979; Kraglund, 1999). In sludge *Ascaris* spp. and *Trichuris* spp. eggs are found to survive for a couple of years, but no surviving eggs are found after 4 years of storage (Schwartzbrod *et al.*, 1986; Ayres *et al.*, 1993). In wastewater and water *A. lumbricoides* and *T. Trichiura* can survive almost a year (Shuval *et al.*, 1986; Feachem *et al.*, 1983).

The most important environmental factor influencing helminth egg/larvae development and survival is temperature (Shuval *et al.*, 1986). The temperature range in which egg development can occur

has been found to be approximately 15-34°C for *A. suum* and *T. suis* (Alicata, 1935; Seamster, 1950; Beer, 1973a; Arene, 1986) and somewhat lower for *Oesophagostomum* spp. eggs from pigs (Fossing *et al.*, 1995; Roepstorff, 1986). Long retention time promotes natural egg die off, however this process can be enhanced with increasing temperatures and decreasing relative humidity. In sludge 99% of *Ascaris* spp. eggs were killed after 97 minutes at 50°C whereas at 20°C a little more than a year was needed for the same result (Pecson *et al.*, 2007), and at 4°C less than 50% of *Ascaris* spp. eggs were killed after 2.5 years (O'Donnel *et al.*, 1984). In slurry 70% of *T. suis* eggs failed to embryonate after 60 minutes at 55°C and over 99% of porcine *Oesophagostomum* spp. eggs were killed after 30 minutes at the same temperature (Burden and Ginnivan, 1978). Many pig *Oesophagostomum* spp. larvae will not survive constant temperatures below 10°C or above 25°C (Roepstorff, 1986; Rose and Small, 1980).

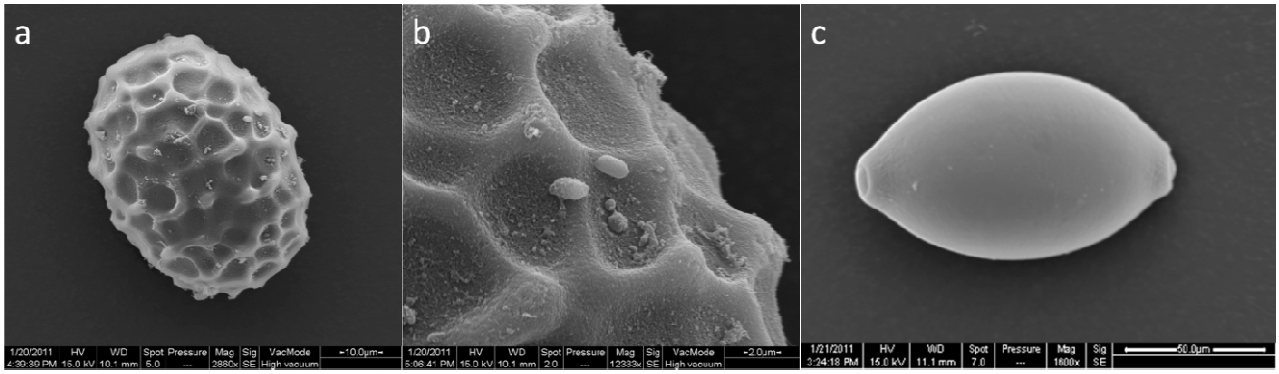

Figure 2.4 Scanning Electron Microscopy photos of *A. suum* (a, b) and *T. suis* (c) eggs stored in 0.1 M sulphuric acid. Photos by the author.

The egg shell of *Ascaris* spp. and *Trichuris* spp. is generally very resistant to most substances with the lipid layer providing the main permeability barrier (Wharton, 1980; Wharton, 1979). The high resistance of *A. suum* and *T. suis* eggs to various chemicals is emphasized by the routine use of sulphuric acid (Fig. 2.4) as embryonation medium (e.g. Oksanen *et al.*, 1990). Because of the high resistance against chemicals *Ascaris* eggs are often used as an indicator organism when evaluating disinfectants and inactivation in wastewater and sludge treatment processes (e.g. O'Donnel *et al.*, 1984; Black *et al.*, 1982). Development and survival of helminth eggs and larvae are also somewhat sensitive to desiccation even though the low permeability of *Ascaris* and *Trichuris* egg shells limits water loss. The rate of water loss is affected by temperature so with prolonged periods of high temperature and/or desiccation the egg will collapse at some point (Wharton, 1980; Wharton, 1979). Due to a thinner shell, eggs of *Oesophagostomum* spp. are not as resistant to environmental stress as eggs of *A. suum* and *T. suis* (Roepstorff, 1986). However *Oesophagostomum* spp. L_3-larvae are

somewhat resistant to desiccation (Goodey, 1924) maybe because of their ability to move in soil. In waste stabilization ponds (see section 2.4.1) hookworm eggs may hatch under aerobic conditions, but since the larvae cannot swim upwards in liquid media they would settle into anaerobic bottom conditions and probably die (Stott *et al.*, 1994). Other environmental factors such as pH and ammonia levels as well as aerobic/anaerobic conditions also affect development and survival rates of helminth eggs in wastewater and sludge (Pecson *et al.*, 2007; Black *et al.*, 1982).

2.1.3 Helminth egg adhesiveness

The eggs of *Ascaris* are widely quoted to be sticky and adhere to surfaces of utensils, vegetables etc. (Raisanen *et al.*, 1985; Gaspard *et al.*, 1994; Jimenez, 2007). It has been proposed that the uterine-derived outer-layer is responsible for the sticky appearance (Fairbairn, 1957). However, there is no evidence for such an egg surface property and the reason for the wide acceptance of eggs being sticky may be that for experimental purposes eggs derived from the female worm uteri are often used. Eggs from the worm uterus have not yet become tanned (see section 2.1.1) and clump together if not treated with sodium hypochlorite, NaClO (e.g. Johnson *et al.*, 1998), or sodium hydroxide, NaOH (e.g. Nejsum *et al.*, 2009). *Ascaris* eggs washed out of feces are tanned (see Fig. 2.1a) and less sticky (Roepstorff, 2003), and would therefore be better representatives of eggs found in the environment with regard to surface properties.

Apart from the attributed sticky appearance of eggs, physical-chemical surface properties are probably involved in adhesion of eggs to surfaces and particles. *Ascaris* eggs were found to have a negative surface charge in water, but the effect of this on particle adhesion is not clear (Dunn, 1991). Stronger adhesion of faeces-derived *A. suum* and *T. suis* eggs to plastic pipettes as compared to glass Pasteur pipettes is observed (observation by the author). The adherence of helminth eggs to surfaces is also addressed in the WHO manual for analysis of helminth eggs in wastewater which recommends rinsing of the plastic containers used to store the wastewater with detergent to release any adhering helminth eggs (Ayres and Mara, 1996). The mechanism and determining factors of helminth egg adherence are not well understood and may, like for other particles, to a large extent depend on the physical-chemical surface properties which are beyond the scope of this thesis. These physical-chemical surface properties are influenced by pH, temperature and the ionic composition of the surrounding water or soil.

2.2 Particles and helminth eggs in water

Particles suspended in water occur either as single entities or in particle flocs/aggregates. The flocculation/aggregation, sedimentation, and overall spatial distribution of particles in water are governed by complex dynamics of physical and chemical forces (Russel *et al.*, 1989). When helminth eggs are discharged into water they can then be viewed as suspended particles and hence it is assumed that the same forces act upon them. In the following section 2.2.1 the forces and processes affecting particles (and thus helminth eggs) in water are described.

In the literature, flocs and aggregates are usually described as different things. Flocs are formed in the water column or on the sediment surface, whereas aggregates are considered to form outside of, and transported to, the aquatic system as stable aggregates (Wall *et al.*, 1978). However, aggregates in the water column will possess many of the same physical, chemical, and biological characteristics as flocs (Droppo, 2001; Droppo and Ongley, 1994). Henceforth the terms floc/flocculation and aggregate/aggregation will be used interchangeably in the thesis.

2.2.1 Suspended particles and flocculation

Fine particles suspended in freshwater stay dispersed because of the dominance of electrostatic repulsion due to the surface charge of particles over the attracting force induced by Van der Waals forces (Russel *et al.*, 1989). Because of the surface charge an electrical double layer exists around each particle in water with the inner layer comprising ions (with opposite charge than the particle) strongly bound to the particle surface while the outer more diffuse layer of ions are more loosely associated (Fig. 2.5) (Elimelech *et al.*, 1995).

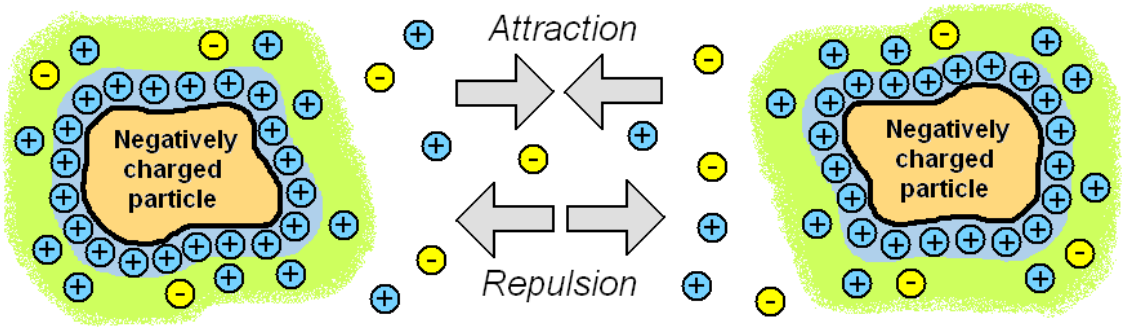

Fig. 2.5 Schematic illustration of the electrical double layer (blue and green areas) around particles in water with positive (blue balls) and negative (yellow balls) ions, and the opposing forces (attraction, repulsion) acting on suspended particles. Illustration by the author.

The repulsive force between particles can be considered as an energy barrier which must be overcome before flocculation/aggregation can take place. This energy barrier is overcome if Van der Waals forces are dominant so particles colliding with each other will stick together. A reduction in the repulsive force can be achieved by different mechanisms, e.g. by changing the ion composition of the water which compresses the double layer around each particle (e.g. by increased salinity, when sea water is mixed with river water (Gibbs, 1983)) or by neutralization of the particle surface charge when oppositely charged polymers (e.g. polysaccharides with biological origin) are adsorbed onto the particle surface (Elimelech *et al.*, 1995). In conventional wastewater treatment addition of charged chemical polymers are often used for enhancing flocculation (see section 2.4.2). The forces acting on suspended particles and the process of flocculation met in natural environments are complex and takes place on different levels. At the same time many of the forces are dependent on the distance between the particles with some forces being very strong at a very small distance, but weak at a larger distance (Elimelech *et al.*, 1995).

Collision between particles can produce flocs. In natural waters, turbulence is the major mechanism facilitating particle collision (Li and Logan, 1997a), but differential settling also plays a large role (Li and Logan, 1997b). Flocs are complex heterogeneous structures typically composed of an active biological component (primarily bacteria), a non-viable biological component (e.g. detritus, extracellular polymeric substances (EPS)), inorganic particles (e.g. clay particles), and water which is held within the floc or flow through pores (Droppo and Ongley, 1994). The threadlike EPS fibrils produced by e.g. bacteria are an important structural component in particle flocs and are responsible for attachment of many cells (Droppo, 2001; Liss *et al.*, 1996). Thus, EPS could play a role in relation to helminth egg adhesion to or flocculation with particles.

2.2.2 Modelling settling velocity

When a particle settles through a fluid it reaches a constant settling velocity when the resistance of fluids (friction) exactly equals the gravitational downward force acting on the particle (Mantovanelli and Ridd, 2006). Settling of particles in water is expected to follow Stokes' law (Stokes, 1851) which is described by the following equation:

$$V_s = \frac{g}{18} \times d^2 \times \left(\rho_p - \rho_l\right) \times \eta^{-1} \qquad \text{(equation 2.1)}$$

where V_s is settling velocity (m s^{-1}), g is gravitational acceleration (9.81 m s^{-2}), d is particle diameter (m), ρ_p is specific density of the particle (kg m^{-3}), ρ_l is specific density of the liquid (kg m^{-3}), and η

is dynamic viscosity of the liquid (kg m^{-1} sec^{-1}). Implicit within Stokes' law are the assumptions that the settling particle has a spherical morphology with a smooth surface and not subjected to aggregation. These assumptions are not always met in nature and high particle concentrations in e.g. wastewater may result in flocculation (Droppo, 2001) which would change the settling behaviour of particles and presumably also helminth eggs.

In the literature, however, settling of pollen (Sosnoskie *et al.*, 2009), bacterial cells (Wan *et al.*, 1995), protozoan (o)ocysts (Medema *et al.*, 1998), as well as helminth eggs (Shuval *et al.*, 1986) has been expected to follow Stokes' law. Theoretical calculations of settling velocities of *A. lumbricoides*, *T. trichiura*, and hookworm eggs in clean water have been made using Stoke's law, i.e. 0.181 mm s^{-1}, 0.425 mm s^{-1}, and 0.108 mm s^{-1}, respectively (Shuval, 1978).

The most significant impact of flocculation is that it alters the hydrodynamic properties of the particles which affect settling processes in the water column. Flocculation may increase the particle size by orders of magnitude over the primary particle size and also affect the shape, density, and porosity (Droppo *et al.*, 1997). The floc shape is also known to affect settling because of the changes in flow resistance causing spherical flocs to generally settle faster than cylindrical and disc shaped flocs (Li and Ganczarczyk, 1987). Floc shape will also influence the spatial orientation of settling and thus the effective vertical settling velocity of the floc. Determination of settling velocity of flocs may also be calculated by Stokes' law if floc size and effective density of the floc are known. Most studies have found that when floc size increases, settling velocity increases too (Kim and Stolzenbach, 2004; Droppo *et al.*, 2000).

Particle transport by gravitational sedimentation is important in almost all water and wastewater treatment processes. Modelling of flocculation as well as transport is required to better understand and predict sediment contaminant transport and wastewater treatment efficiency (Johnson *et al.*, 1996).

2.3 Resuspension of sediment and helminth eggs

When helminth eggs are removed from water by sedimentation and deposited on the sediment bed they accumulate in the sediment or sludge, and this represents a potential health hazard if the sludge is handled and used as crop fertilizer (Nelson, 2003). Resuspension of deposited helminth eggs from sediments in e.g. irrigation canals or in natural ponds will lead to increased concentration of

suspended eggs in the water. Moving water will apply a force on the sediment bed, the bed shear stress, usually expressed in N m^{-2} (Black *et al.*, 2002). The bed shear stress acting on a particle resting on the bed is counteracted by the particle weight, aggregation with other particles, and cohesion (see section 2.3.1). Resuspension or erosion of sediment is induced when the bed shear stress exceeds the stabilizing forces. This threshold for erosion of sediments is denoted critical bed shear stress or simply erosion threshold. Erosion usually occurs due to increased hydrodynamic impact induced by e.g. increased current speed, surface waves or human disturbances like collection or pumping of water for irrigation.

2.3.1 Sediment and helminth egg erosion potential

Cohesive sediment, or mud (here defined as particles with a grain size smaller than 63µm), is a mixture of clay particles, silt, organic material, and a large amount of water and is encountered in most water bodies throughout the world (Droppo, 2001). This sediment has cohesive properties because of the electrochemical attraction of clay particles and organic material and will therefore form aggregates (Eisma, 1986). Direct or indirect particle aggregation may also take place due to presence of microphytobenthos (Paterson, 1989; Yallop *et al.*, 1994) and bacteria (Grant and Gust, 1987) producing EPS (section 2.2.1) which will create bonds between bed-particles (Fig. 2.6). Macro-fauna will tend to aggregate cohesive sediment through feeding or movement activities (Andersen, 2001; de Brouwer *et al.*, 2000; de Deckere *et al.*, 2000), but destabilize the sediment through bioturbation activities, e.g. burrowing and sediment pelletization (Andersen and Pejrup, 2002; Andersen *et al.*, 2005). The erodibility or erosion potential (defined as the ease with which particles are removed from the bed surface and put into suspension again) of cohesive sediment beds will typically show strong dependence on these physical and biological factors and processes, i.e. a dependence on grain size composition (e.g. mud content), bed roughness, and biological activity (Black *et al.*, 2002).

Fig. 2.6 Biofilm at an intertidal mudflat. Photo: Thorbjørn J. Andersen.

Sediment cohesion and aggregation has a marked effect on both the erosion potential and settling velocity of the sediment, typically resulting in increased erosion resistance of sediment beds and increased settling velocity of suspended sediment (Andersen and Pejrup, 2002; Nowell *et al.*, 1981). Both physical and biological properties of sediments may display seasonal variation, and hence temporally varying erosion potential and settling velocity (Andersen, 2001). Sediment particles deposited on fine-grained beds in nature are incorporated into the sediment bed over time due to consolidation and the range of biological and physical-chemical processes mentioned above. If and to what extent helminth eggs take part in the aggregation of fine-grained particles in nature is unknown. If helminth eggs adhere to suspended particles and are part of flocculation processes it may have implications for resuspension of helminth eggs. Egg-entrapment in settling particle flocs may cause better incorporation into the sediment bed and thus decrease the erodibility of the eggs. The magnitude of egg incorporation may also depend on the sediment composition and incorporation time.

2.4 Reducing helminth egg numbers in wastewater

The occurrence and concentration of helminth eggs in wastewater depend on several factors, e.g. the helminth species, the number of infected people serving as the source, and the volume and concentration of sewage (Shuval *et al.*, 1986). Generally, *Ascaris* eggs are much more common in wastewater and sludge than eggs of *Trichuris* and hookworm, probably because the female worm of *Ascaris* can produce up to 20,000 eggs per day (Feachem *et al.*, 1983). The concentration of *Ascaris* in raw wastewater may vary from 10-100 eggs per L in endemic areas to 100-1,000 eggs per L in hyperendemic areas (Kamizoulis, 2008; Mara and Sleigh, 2010). Bhaskaran *et al.* (1956) found concentrations of 200-2,000 *Ascaris* eggs, 20-800 hookworm eggs, and 10-30 *Trichuris* eggs per L sewage from Calcutta, India. Even in countries thought to have low incidences, like England *Ascaris* eggs were found at a concentration of 200 eggs per L wastewater (Dunn, 1991). Also in France both *Ascaris* and *Trichuris* eggs were recovered from sludge (Schwartzbrod *et al.*, 1986) and in Barcelona, Spain helminth egg concentrations varied from 20 to 340 eggs per kg sludge (Schwartzbrod *et al.*, 1989).

2.4.1 Helminth egg removal in wastewater treatment

Helminth egg removal is commonly assessed by comparing the number of eggs present in influent and effluent wastewater. Removal rates may thus only refer to the removal of eggs from suspension in wastewater into sediments and sludge, and not to the inactivation of eggs (Stott, 2003). In

relation to inactivation, assessment of the viability is usually determined based on a morphological observation of egg development (Johnson *et al.*, 1998). Further, egg viability does not necessarily imply that eggs are infective which can only be determined by experimental infection studies.

Fig. 2.7 Conventional wastewater treatment, 'Lynetten', Copenhagen, Denmark
Photo by Lynetten (http://www.lyn-is.dk/)

Fig. 2.8 Waste stabilization ponds in Arusha, Tanzania. Photo by Arusha Urban Water Supply and Sewerage Authority (http://www.auwsa.or.tz/index.php/gallery/)

Wastewater can be treated by a variety of methods from conventional, highly mechanized processes (Fig 2.7) to simple, more natural systems (Fig 2.8). In most of these treatments helminth eggs are removed by coagulation-flocculation processes followed by sedimentation, although there is a wide variation in removal efficiency (Scheierling *et al.*, 2010). Conventional treatment has shown to significantly reduce helminth egg concentrations. Bhaskaran *et al.* (1956) reported 82-100% removal of *Ascaris*, *Trichuris*, and hookworm eggs by secondary wastewater treatment, i.e. activated sludge and trickling filters. When applying sand filtration (tertiary treatment) almost 100% removal has been obtained for helminth eggs (Schwartzbrod *et al.*, 1989; Rose *et al.*, 1996). However, conventional wastewater treatment is costly, uses advanced technology and requires a high level of maintenance. Such treatment technologies therefore often fail when installed in low-income countries (Kim and Stolzenbach, 2004).

Several natural wastewater treatment systems exist with waste stabilization ponds (WSPs) being one of the most well-documented appropriate wastewater treatment technologies in low-income countries (Mara, 2009). WSPs can effectively remove helminth eggs from wastewater with the principal removal mechanism being sedimentation facilitated by long retention time (Mara *et al.*,

1992; Mara, 2004), although hookworm larvae have been found in the final pond effluent (Stott *et al.*, 1994; Ellis *et al.*, 1993). A study with single ponds (unknown retention times) reported helminth egg removal rates of 63-93% (Verannan, 1977) whereas Mara and Silva (1986) showed that a retention time of 11 days in a three-pond series (anaerobic, facultative and maturation) could achieve the WHO water reuse guidelines of ≤1 egg per litre (WHO, 2006). Further, a model was developed for predicting nematode removal as a function of hydraulic retention time based on the initial concentrations of nematode eggs in raw wastewater (Ayres *et al.*, 1992). Using this model it was estimated that a retention time of nine days would remove up to 99% of the eggs.

2.4.2 Assisted sedimentation using coagulants

Chemically assisted sedimentation using coagulants (ferric chloride, alum, or lime) creating larger particle flocs with higher settling velocity than the particles alone is normal procedure in most conventional wastewater treatment (AWWA, 1990). Apart from particle and turbidity removal from wastewater chemical coagulation has also proven efficient in facilitating removal of helminth eggs. In a study from Mexico, advanced primary treatment used aluminium sulphate as coagulant followed by sedimentation and resulted in helminth egg removal rates of 95% (Jimenez *et al.*, 2000). The cost involved in achieving the desired level of treatment depends among other things, on the cost and availability of chemicals. Conventional treatment technologies often are not feasible in low-income countries. Additionally, many of the chemicals are associated with human health and environmental problems (AWWA, 1990; Kaggwa *et al.*, 2001).

2.4.3 Coagulation by Moringa oleifera seed extracts

Natural coagulants of plant origin have been used in water treatment since the advent of the chemical salts, but they have not been able to compete due to lack of scientific evidence of their effectiveness on e.g. helminth eggs. One of the natural coagulants of main interest is extracts of the dry seed of *Moringa oleifera* (MO; Fig 2.9), which increasingly have been recognized as a cheap substitute in wastewater treatment (Sutherland *et*

Fig. 2.9 *Moringa oleifera* tree with pods containing seeds which extracts are used as natural coagulants. (Photo from http://davesgarden.com/)

al., 1989; Jahn, 1988). *Moringa* is a tropical plant belonging to the family of *Moringaceae*, where MO is the most widespread species and is non toxic to humans and animals (Berger *et al.*, 1984). MO trees grow quickly and are known for their medicinal use and source of nutrition.

It is documented that MO seed extracts are efficient in reducing the turbidity of water (Muyibi and Alfugara, 2003; Ndabigengesere and Narasiah, 1998; Boateng, 2001). As mentioned in section 2.2.1, suspended particles can stay dispersed due to the electrostatic repulsion (negative) between particles. By adding cationic proteins extracted from dry MO seeds the electric charge of particles is neutralized and hence particles begin to flocculate due to Van der Waals attractive forces or turbulence (Jahn, 1988; Muyibi and Evison, 1995; Folkard and Sutherland, 2002). It is believed that a combination of different mechanisms is causing coagulation to take place. One mechanism is that positively charged MO proteins bind to the negatively charged suspended particles by Coulomb forces, thus neutralizing the surface charge and the resulting reduced electrostatic repulsion leads to flocculation of particles. Another mechanism is that positively charged MO proteins bind to parts of the surface of the negatively charged particles (by adsorption) and flocs are formed due to particle collision and interparticulate saturation of the differently charged particle surface areas (Bhatia *et al.*, 2007a; Bhatia *et al.*, 2007b; Gassenschmidt *et al.*, 1995; Ndabigengesere *et al.*, 1995). In high turbid water there are an increased number of suspended particles available for adsorption and colloidal charge neutralization. The net effect is an increase in particle collision frequency and formation of flocs which settle faster than the colloids alone (LaMer and Healy, 1963; Birkner and Morgan, 1968). Studies have shown that treatment of water with MO seed extracts can achieve 90-99.99% reduction in pathogens, i.e. fecal bacteria and *Schistosoma mansoni* cercariae (Olsen, 1987; Madsen *et al.*, 1987). It is however unknown if coagulation and flocculation processes induced by MO has an effect on helminth egg numbers in different types of water.

Chapter 3

Materials and methods

Three sub-studies were carried out to meet the objectives of the PhD project (see section 1.1). The first study focused on determining the settling velocity of helminth eggs and on their interaction with suspended wastewater particles. The second study investigated resuspension of helminth eggs after sedimentation and interaction with cohesive sediment and suspended particles. The last study explored to which extent the settling velocity of helminth eggs could be enhanced by seed extracts of *Moringa oleifera* (assisted sedimentation), thereby introducing a low technology and environmentally friendly water treatment method for helminth egg removal. The present chapter gives an overview of the methods used in the PhD project. The reader is referred to the three specific papers in Chapter 4 for a comprehensive description of the methods and materials used in each experiment.

3.1 Helminth egg material (Paper I, II, and III)

Eggs of *Ascaris suum*, *Trichuris suis*, and *Oesophagostomum* spp. were recovered from fresh faeces of naturally infected pigs in Denmark. The method used for isolating helminth eggs from faeces consisted in a combination of sieving (Jørgensen, 1978; Oksanen *et al.*, 1990) and flotation (Larsen and Roepstorff, 1999; Roepstorff and Nansen, 1998). For sieving, a series of sieves with decreasing mesh sizes was used (Table 3.1). Subsequently, flotation of eggs was done using flotation fluid (50

Table 3.1 Sieves used for isolating helminth eggs from faeces.

	A. suum	*T. suis*	*Oesophagostomum* spp.
Mesh size (µm)	500	500	500
	212	212	212
	90	90	90
	38	38	53
	20	35	20
		31.5	
		30	
		20	

g glucose monohydrate/100 ml saturated NaCl solution yielding a density of 1.27 g/ml) with higher density than the eggs. Eggs of *A. suum* and *T. suis* were stored in H_2SO_4 (0.05M, pH 1, 24 eggs/µl), and *Oesophagostomum* spp. eggs in demineralized water (24 eggs/µl) at 5°C (Eriksen, 1990). All helminth eggs used in the three main experiments were isolated as described above.

3.2 Sedimentation experiments (Paper I)

To determine the settling velocity of suspended helminth eggs and particles sedimentation experiments were carried out with a settling tube, i.e. an Owen tube (Owen, 1976). This method is commonly used in sediment research in freshwater, estuarine, and marine waters (e.g. Dyer *et al.*, 1996; Dearnaley, 1996; Pejrup, 1988). The method was modified and optimized for determination of helminth egg settling velocity with regard to Owen tube construction, time intervals, and number of helminth eggs.

Fig. 3.1 The Owen tubes used for sedimentation experiments.

3.2.1 The Owen tube method

Owen tubes were constructed in acrylic plastic (Fig. 3.1). The basic operation is to fill the tube with water and allow the particles and helminth eggs (500-600 eggs) to settle. Samples are then taken from the bottom of the tube by a funnel which is closed with a clamp. Ten subsamples are manually obtained at predetermined time intervals until the tube is emptied. Subsequently, particle concentration and the number of eggs in each subsample are quantified (see section 3.5 and 3.6, respectively) and used for calculation of the corresponding settling velocity. The settling velocity was determined for helminth eggs in tap water and in wastewater as well as for particles in wastewater.

A comprehensive description of Owen's settling velocity calculation method is found elsewhere (Owen, 1976). The calculation of the settling velocity is based on the measured differences in egg numbers (or particle concentration) in the ten sub samples (from one Owen tube experiment), the predetermined time intervals, and the total settling height in the tube (1 meter) as

well as the temperature (Pejrup, 1988). The calculated settling velocity represents the median settling velocity (mm s^{-1}) of all the eggs or particles in that experiment.

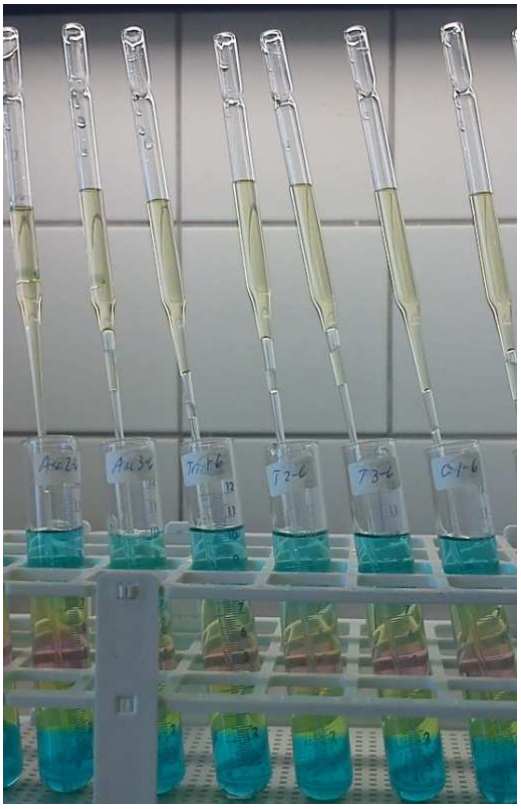

Fig. 3.2 Density gradient centrifugation. Layers of sucrose-solution with different densities. Helminth eggs placed on the top layer moves downward when centrifuged.

3.2.2 Theoretical settling velocity of helminth eggs

For theoretical calculation of helminth egg settling velocity as predicted by Stokes' law the different parameters in Stokes' equation (see equation 2.1, page 17) were determined. The mean egg sizes (*d*) of the three egg types (*Ascaris*, *Trichuris* and *Oesophagostomum*) were measured (see section 3.7). The density (ρ_p) of each egg type was determined by density grading centrifugation (Fig. 3.2; (David and Lindquist, 1982)), whereas the density of tap water (ρ_l) was measured with a pycnometer (Fig. 3.3). The kinematic viscosity (η) of tap water was measured with a viscometer. For Stokes' equation the dynamic viscosity (η) was calculated from the kinematic viscosity (ν) using the following equation: $\eta = \nu \times \rho$, where η is the dynamic viscosity (kg m^{-1} sec^{-1}), ν is the kinematic viscosity (m^2 s^{-1}), and ρ is the density of the medium (kg m^{-3}). The calculated settling velocities of the three types of helminth eggs were compared to the settling velocities obtained from Owen tube experiments with tap water containing eggs.

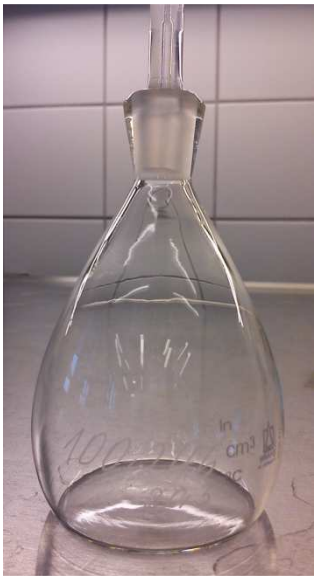

Fig. 3.3 Pycnometer for density measurement of liquids with a known volume (cm^3) used to measure the mass (g).

3.3 Resuspension experiments (Paper II)

Resuspension or erosion of sediments is widely investigated in freshwater, estuarine, and marine sediment research in order to predict sediment transport, e.g. of hazardous contaminants like heavy metals accumulated in sediments. Different methods are applied both in field and laboratory trials. In the present study a portable version of the German EROMES erosion equipment (Schünemann and Kühl, 1991;

Andersen, 2001) was used (section 3.3.1). The method was modified and optimized for determination of both helminth eggs and sediment erosion with regard to sediment bed type, number of helminth eggs, time intervals, and applied bed shear stress.

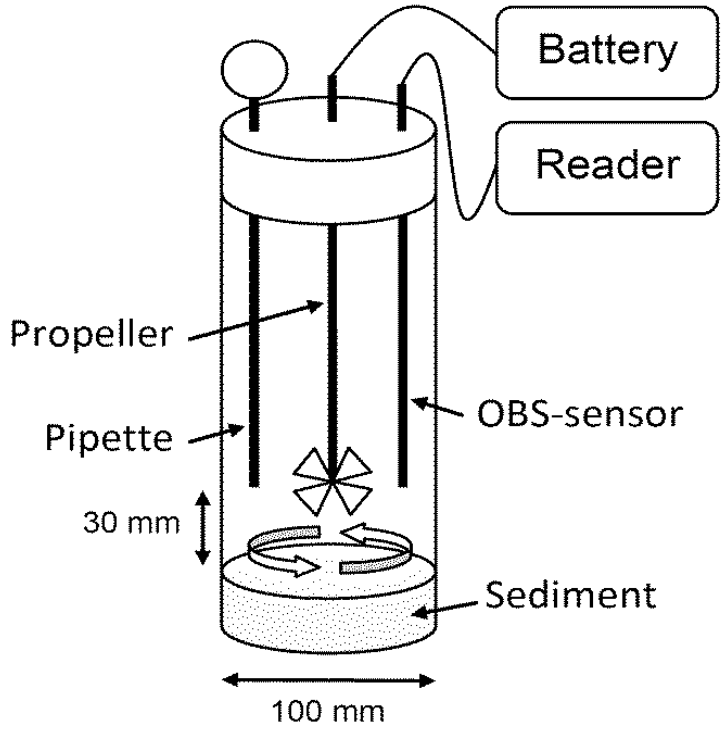

Fig. 3.4: Schematic illustration of the erosion device used for determination of erosion rates and erosion threshold.

3.3.1 Erosion experiments

For determination of helminth egg resuspension (i.e. erosion rate and erosion threshold) experiments were carried out with the erosion device shown in Figure 3.4. A sediment bed was prepared with natural sediment and water from Tibberup freshwater stream (Northern Sealand, Denmark), and *Ascaris* and *Trichuris* eggs (approximately 12,000 of each egg type per litre) were added to the water and allowed to settle with gravity onto the sediment bed. Bed shear stress was generated by a propeller induced flow and the suspended particulate matter concentration (SPMC) was monitored by an optical back-scattering (OBS) sensor. The bed shear stress was increased every six minutes (0, 6, 12, 18, 24, 30, and 36 min) from 0 N m^{-2} to 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 N m^{-2}. The OBS readings where calibrated by SPMC measurements. Prior to every increase in bed shear stress subsamples were collected for quantification of helminth egg numbers and SPMC (see section 3.5 and 3.6, respectively). Identical erosion experiments were carried out on day 1, 2, and 3 after bed preparation in order to investigate a possible time effect on the erodibility of sediment and helminth eggs.

3.3.2 Settling velocity experiment and calculation

Aggregation and settling velocity of both the resuspended material and helminth eggs were investigated as part of the erosion experiments. After the propeller was turned off at the maximum bed shear stress of 0.5 N m^{-2} sediment and eggs were allowed to settle for six minutes. The change

in SPMC and egg numbers was monitored continuously by OBS readings every 30 seconds and subsamples collected every minute for quantification of SPMC and helminth egg numbers (see section 3.6 and 3.5, respectively). The results from the subsamples were then used for calculation of settling velocity of both the sediment and helminth eggs. Additionally, the settling velocity of helminth eggs in clean tap water was determined as controls with the same apparatus and method as mentioned but without sediment.

The settling velocity of helminth eggs and particles was calculated using the method of Amos and Mosher (1985). This calculation method is different from the Owen method described above due to the different experimental setup. Prior to calculation of settling velocities a plot of SPMC or egg numbers (on log-scale) as a function of settling time was done for graphical inspection of the straight line of the exponential function. Mean values of SPMC and egg numbers for three experimental cores per day were plotted (see additional results, section 4.2.1). The settling velocity (V_0) was calculated using the following equation:

$$V_0 = \frac{-\ln(0.5)}{p t_{½}} \times y \qquad \text{(equation 3.1)}$$

where y is the depth at which the settling velocity is evaluated and p is the probability of grain deposition (1 in this setup with stagnant water). The time over which 50% of the particles or helminth eggs settle are denoted $t_{½}$ and is found by:

$$t_{½} = \frac{\ln(0.5)}{x} \qquad \text{(equation 3.2)}$$

where x is the regression coefficient found by plotting the SPMC or number of eggs as a function of settling time and fitting data with a exponential function.

3.3.3 Sediment properties

Sediment stability or erosion potential is affected by a number of physico-chemical and biological properties of the sediment (see section 2.3.1) which were characterized for each of the nine sediment cores in the experiment. As for the physico-chemical properties, grain size distribution, water content, dry bulk density, and organic content were determined. The number of bacterial cells, and the content of chlorophyll *a* and its degradation product phaeopigment were measured as the biological sediment properties. The chlorophyll *a* content was used as an indicator of the amount of living diatoms.

3.4 *Moringa*-assisted sedimentation experiments (Paper III)

To determine if the settling velocity of helminth eggs could be enhanced by assisted sedimentation experiments were carried out with seed extracts of *Moringa oleifera* (MO) which is a natural coagulant (see section 2.4.3). With the scope of introducing a low technology treatment method for helminth egg removal, experiments were carried out both under field conditions in Ghana (performed by B. Keraita and O.K. Boateng) and under laboratory conditions in Denmark (performed by the author). In Ghana, helminth eggs were present in irrigation water contaminated with untreated domestic wastewater. In Denmark, 2,100-2,200 eggs of each type of helminth (*Ascaris* and *Trichuris*) per litre were added to the different water types used for the laboratory experiments.

3.4.1 Study sites and water types

The field experiments were done at Karikari, a prominent urban vegetable farming site in Kumasi, Ghana. Samples of irrigation water were taken from on-farm ponds (dug-outs) which receive wastewater from nearby households and were used for irrigation at the time of the study. Laboratory experiments were conducted to model field conditions where water used for irrigation may show high variation in pollution levels, e.g. due to changes in water turbidity. Three water types with low turbidity (< 50 NTU; tap water), medium turbidity (50-150 NTU; wastewater) and high turbidity (>150 NTU; model turbid water), respectively were used (Lea, 2010).

3.4.2 Extraction of coagulant and determination of optimum dosage

MO seeds used for coagulation in both field and laboratory experiments were obtained from a commercial seed supplier in Kumasi, Ghana. The shell was removed and the MO seeds (Fig 3.5) were manually grounded to a fine powder and mixed with tap water (Ndabigengesere and Narasiah, 1998) to reach a final concentration of 3% or 5% weight per volume (w/v) suspension. The optimum MO extract dosage was determined using the jar test (see section 3.4.3) by adding different volumes of the 3% or 5% MO extract. The optimum dosage of MO extract was identified as the

Fig. 3.5. *Moringa oleifera* seeds with and without the shell.

volume and concentration level above which no further reduction in turbidity was observed when more MO extract was added.

3.4.3 Jar test and experimental design

The principle in the jar test is that a series of jars each representing a predetermined time point is filled with one liter of water. Subsequently, the optimum dosage of MO extract (MO+) is mixed with the water and the jar left for coagulation and sedimentation to take place. Some jars with no MO extract added (MO-) are used as controls. At predetermined time intervals the jars are taken out of the experiment and the supernatant (800 ml) collected. In the supernatant the number of helminth eggs is counted (see section 3.5) and the turbidity determined (see section 3.6).

3.4.4 Enhanced settling velocity

The number of helminth eggs and the turbidity in each jar corresponding to a certain time point was used to calculate the settling velocity of suspended eggs and particles as done in section 3.3.2. To quantify a possible enhanced sedimentation, calculation of median settling velocities with and without MO extract was done for settling times of 15 min and 60 min in the laboratory experiments (see additional results, section 4.3.2).

3.5 Enumeration of helminth eggs in water samples (Paper I, II, and III)

Water samples containing helminth eggs were centrifuged and the supernatant discarded in order to concentrate the eggs in a small volume of water. Flotation fluid (50 g glucose monohydrate/100 ml saturated NaCl solution yielding a density of 1.27 g/ml) was added and the helminth eggs counted in McMaster chambers. Water samples containing many particles were poured through one layer of gauze and rinsed well prior to centrifugation (**Paper I** and **III**). A detergent solution, 0.01% Tween20 solution, was used to rinse centrifuge tubes and other laboratory utensils to ensure that all eggs were transferred (Ayres and Mara, 1996). Optimization of the egg counting method was performed with regard to the material (glass or plastic) of both centrifuge tubes and pipettes, filtering of samples with many particles (number of gauze layers and rinsing steps), use of detergent solution (water or Tween20 solution), and counting by sedimentation or flotation (Sedgwick-Rafter or McMaster chambers).

In the MO experiments in Ghana, helminth eggs were enumerated using the U.S. Environmental Protection Agency (USEPA) modified concentration method (Schwartzbrod, 1998). In this method flotation of helminth eggs using $ZnSO_4$ solution (density of 1.2 g/ml) is performed followed by sedimentation and concentration prior to counting of egg numbers in Sedgwick-Rafter chambers.

3.6 Determination of particle concentration in water samples (Paper I, II, and III)

The particle concentration in water samples was determined either by dry weight (paper I and II) or by light back-scattering (**Paper II** and **III**). The principle of the dry weight method is to filter water samples (multiple filter sizes in **Paper I**), dry the filters (Fig. 3.6) and determine the dry weight of the particles from the sample by comparison with control filters processed with clean water (Pejrup, 1988; Jones and Jago, 1996). Optimization of this method was performed with regard to filter size, and number of filters.

Fig 3.6 Series of dried filters showing increasing particle concentration ready for weighing. Three control filters are seen in the lower row to the right.

The second method makes use of the fact that suspended particles will scatter incident light in all directions and that the amount of scattered light will depend on the concentration of particles. The particle concentration is referred to as turbidity (fluid cloudiness) and is measured in Nephelometric Turbidity Units (NTU) with an optical back-scattering (OBS) sensor (**Paper II**) or a portable turbidimeter (**Paper III**). In both cases a light beam is sent into the water sample and a detector (photodiode) quantifies the light back-scattering. The higher particle concentration in the water the more light is scattered and reaches the detector and the higher the NTU reading.

3.7 Size distribution of helminth eggs and particles (paper I, II and III)

The sizes of *A. suum, T. suis* and *Oesophagostomum* spp. eggs were determined by measuring the largest diameter (length) and the smallest diameter (width) of 100 eggs of each helminth under a microscope equipped with a camera. The mean values of the length and the width for each set of samples were used to calculate a mean egg size of each helminth type used in Stokes' equation (**Paper I**). Additionally, the egg size distribution of each helminth egg type was measured with a laser particle analyzer (LISST 100C; (Agrawal and Pottsmith, 2000) by analyzing the light diffraction of egg solutions (0.5-1.5 million eggs in tap water; **Paper I**). The particle size distribution of primary sediment particles (**Paper II**) and of primary particles in turbid water and wastewater (see additional results, section 4.3.2) was determined by laser-sizing using a Malvern Mastersizer 2000 after addition of 0.01M $Na_2P_4O_7$ and sonification.

3.8 Statistical analysis (paper I, II, and III)

Log-transformed and non-transformed data with a normal distribution were analyzed by ANOVA analyses. Depending on the number of fixed effects, one-way analysis was performed on settling velocities from the sedimentation experiment (**Paper I**), two-way analysis for the effect of MO on helminth eggs and turbidity (**Paper III**), two-way analysis for the effect on MO on settling velocities of helminth eggs and particles (additional results, section 4.3.2), and three-way analysis on settling velocities from the resuspension experiment (**Paper II**). Erosion rates were analyzed by an ANOVA with time (shear stress) as repeated measures (**Paper II**). For all ANOVA analyses, the non-significant effects were removed by backward model reduction on a 5% significance level, and post-hoc tests with Tukey-HSD correction for multiple testing were done when interactions were significant. In the MO experiments (**Paper III**) linear regression was performed for helminth egg number as a function of turbidity for irrigation water, turbid water, and wastewater.

Chapter 4

Sub-studies: results and discussion

Results and discussion of findings in the three sub-studies are presented in the following sections as **Paper I**, **Paper II**, and **Paper III**. Additional results and discussion not presented in the papers from the resuspension experiment (**Paper II**) and from the *Moringa*-assisted sedimentation experiment (**Paper III**) are included in this chapter following the two papers.

4.1 Paper I

Sedimentation of helminth eggs in water

Sengupta, M. E., Thamsborg, S. T., Andersen, T. J., Olsen, A., and Dalsgaard, A. 2011. Sedimentation of helminth eggs in water. Water Research 45:4651-4660

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Sedimentation of helminth eggs in water

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ABSTRACT

Helminth parasite eggs in low quality water represent health risks when used for irrigation of crops. The settling velocities of helminth eggs (*Ascaris suum*, *Trichuris suis*, and *Oesophagostomum* spp.) and wastewater particles were experimentally determined in tap water and in wastewater using Owen tubes. The settling velocities of eggs in tap water was compared with theoretical settling velocities calculated by Stoke's law using measurements of size and density of eggs as well as density and viscosity of tap water. The mean settling velocity in tap water of 0.0612 mm s^{-1} found for *A. suum* eggs was significantly lower than the corresponding values of 0.1487 mm s^{-1} for *T. suis* and 0.1262 mm s^{-1} for *Oesophagostomum* spp. eggs. For *T. suis* and *Oesophagostomum* spp. eggs the theoretical settling velocities were comparable with the observed velocities in the Owen tubes, while it was three times higher for *A. suum* eggs. In wastewater, the mean settling velocity for *A. suum* eggs (0.1582 mm s^{-1}) was found to be different from *T. suis* (0.0870 mm s^{-1}), *Oesophagostomum* spp. (0.1051 mm s^{-1}), and wastewater particles (0.0474 mm s^{-1}). This strongly indicates that in low quality water the eggs are incorporated into particle flocs with different settling velocities and that the settling velocity of eggs and particles is closely associated. Our results document that there is a need to differentiate the sedimentation of different types of helminth eggs when assessing the quality of low quality water, e.g. for irrigation usage. The results can also be used to improve existing models for helminth egg removal.

1. Introduction

Freshwater sources are becoming increasingly scarcer in many parts of the world and use of wastewater has become an attractive option for conserving and expanding available water supplies. Wastewater use in agriculture has a long history and it has been estimated that about 20 million hectares of agricultural land worldwide is irrigated with treated or untreated wastewater (Jimenez and Asano, 2008). Especially when untreated wastewater is used for crop irrigation, it poses substantial risks to human health, not only for farmers, but also for surrounding communities and consumers of the crops, in particular when crops are eaten uncooked. The major health risks from irrigation with low quality water are associated with viral, bacterial, and parasite pathogens that are usually present in untreated, partially treated, and occasionally treated wastewater (Feachem et al., 1983; Shuval et al., 1986).

Helminth parasite eggs in wastewater are of particular health concern due to the high burden of helminthic diseases

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in low-income and lower-middle-income countries where use of wastewater is most prominent. The most important intestinal worms are *Ascaris lumbricoides* (the human roundworm), *Trichuris trichiura* (the human whipworm), *Ancylostoma duodenale* and *Necator americanus* (the two human hookworms), and *Taenia saginata* and *T. solium* (the beef and pork tapeworms) (Feachem et al., 1983; Shuval et al., 1986). It is estimated that ascariasis globally affects more than 1.2 billion people while trichuriasis and hookworm each affect 700–800 million people (Bethony et al., 2006; de Silva et al., 2003). The helminth eggs are shed by infected humans within whom the female worms produce vast quantities of eggs (up to 200,000 per day) (Feachem et al., 1983). The eggs enter the wastewater through direct fecal input, discharge of treated and untreated sewage, and surface water runoff from agricultural lands (Kirby et al., 2003). Since helminth parasites are extremely resistant to environmental stress, a high degree of helminth egg removal is required by wastewater treatment processes if treated wastewater is to be used safely in irrigated agriculture (Stott, 2003). In its recent guidelines the World Health Organization (WHO) therefore recommends that wastewater used for irrigation should contain less than one helminth egg per liter when no other risk reduction options are available (WHO, 2006).

The occurrence and concentration of helminth eggs in wastewater depend on a variety of factors, i.e. the parasite species, the number of infected persons serving as the source, and the volume and concentration of sewage (Shuval et al., 1986). The concentration of *Ascaris* in raw wastewater may vary from 10–100 eggs per L in endemic areas to 100–1000 eggs per L in hyperendemic areas (Kamizoulis, 2008; Mara and Sleigh, 2010). Wastewater can be treated by a variety of methods involving highly mechanized processes, from secondary and tertiary treatment, to simple waste stabilization ponds (WSP; anaerobic, facultative, and maturation). In most of these treatments helminth eggs are removed by sedimentation processes, although there is a wide variation in removal efficiency (Shuval et al., 1986). Tertiary treatment can significantly reduce helminth egg concentrations, but it is costly, uses advanced technology and requires a high level of maintenance. Such treatment technologies therefore often fail when installed in low-income countries (Kim and Stolzenbach, 2004). Waste stabilization ponds on the other hand are well-documented appropriate wastewater treatment technologies in low-income countries and have demonstrated high rates of helminth egg removal, although hookworm larvae have been found in the final pond effluent (Mara, 2004; Ellis et al., 1993; Stott et al., 1994). Among the wastewater treatment systems, WSPs have been designed specifically for helminth egg removal. A model was developed by Ayres et al. (1992) for predicting nematode egg removal as a function of hydraulic retention time, when effluent is required for restricted irrigation only. Based on the initial concentrations of nematode eggs in raw wastewater this model can be used to determine the retention time in different pond types (anaerobic, facultative and maturation) needed to treat wastewater so it meets the WHO water quality guideline value of less than one helminth egg per L (Ayres et al., 1992). Using this model it was estimated that a retention time of nine days would remove up to 99% of the eggs, but the model does not include parameters such as water depth, temperature, and turbulence, which are all likely to influence the sedimentation rate of parasite eggs. However, series of pond types are more efficient than fewer ponds with long retention times. Since helminth eggs remain viable for several months and even years in the environment (Feachem et al., 1983), accumulated eggs in sludge and other types of sediments represent a health hazard when the sludge is handled and used as crop fertilizer. Further, resuspension of parasite eggs in sediments, e.g. in wastewater and irrigation canals, during increased water flow and disturbance events like storm water and when pumping water, will lead to increased concentration of eggs in water and associated health risks when such water is used in irrigated agriculture. However, little is known about resuspension processes of helminth eggs under different hydrologic conditions in aquatic environments.

Sedimentation of particles in water is expected to follow Stokes's law which implies that settling velocity depends on particle size (defined as spherical), difference in density between particles and water, and the viscosity of water. This is also assumed for pollen (Sosnoskie et al., 2009), bacterial cells (Wan et al., 1995), protozoa (oo)cysts (Medema et al., 1998), and helminth eggs (Shuval et al., 1986). Theoretical calculations of the settling velocities of *Ascaris*, *Trichuris*, and hookworm eggs in clean water have been made by using Stoke's law, i.e. 0.65 $m\,h^{-1}$, 1.53 $m\,h^{-1}$, and 0.39 $m\,h^{-1}$, respectively (Shuval, 1978). However, these calculations do not take into account differences in morphologies and densities of the different types and development stages of helminth eggs and have not been confirmed experimentally. Further, high particle concentrations in wastewater typically results in flocculation of the particles (Droppo, 2001) and this may lead to attachment and entrapment of helminth eggs to these flocs affecting the settling velocity of the eggs. Flocculation generally increases settling velocity of the suspended particles due to the increase in apparent particle diameter. This has been documented for protozoan parasites attached to wastewater particles (Medema et al., 1998). However, floc density at the same time decreases when water makes up part or most of the flocs and if these have very low densities, the outcome may actually be a decrease in settling velocity. This effect of decreased settling velocity of particle flocs in water, however, has not yet been studied.

The pig helminths, *Ascaris suum*, *Trichuris suis*, and *Oesophagostomum* spp. (from now on referred to as Ascaris, Trichuris and Oseophagostomum) are often used as model organisms for the previous mentioned human helminth parasites (Boes and Helwigh, 2000). The eggs of pig helminths are virtually identical in morphology and size to the corresponding human parasite eggs and are relative easy to obtain in high numbers from infected pigs. The conformation of Ascaris, Trichuris and Oesophagostomum eggs are very different from spherical particles (Fig. 1). The Ascaris egg is round to elliptical in shape, covered with a proteinaceous external layer which is irregularly mammillated. Trichuris eggs are lemon shaped with a knob at each pole (polar plugs) and have a smooth surface whereas the egg of Oesophagostomum is elliptical with a smooth surface (Alicata, 1935). The eggs of Ascaris and Trichuris are quoted to be sticky and adhere to surfaces and suspended matter, e.g. in wastewater (Gaspard et al., 1994; Roepstorff, 2003). A small negative

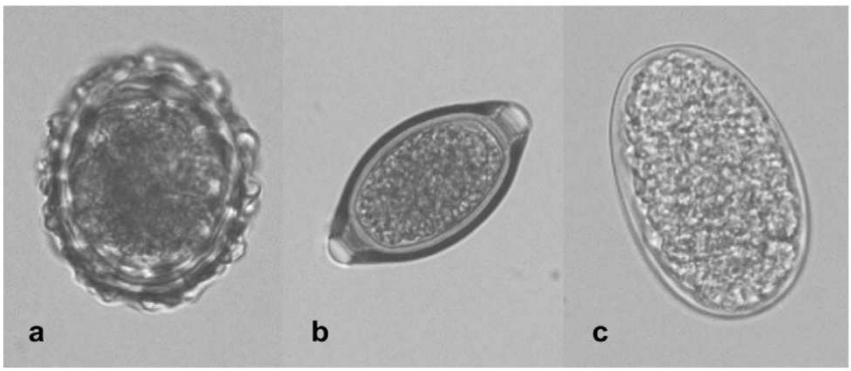

Fig. 1 – Morphology of the three helminth eggs used in the sedimentation experiments. a. *Ascaris suum*; b. *Trichuris suis*; c. *Oesophagostomum* spp. Not to scale. Photo © Mita E. Sengupta.

surface charge has been reported for Ascaris eggs (Capizzi and Schwartzbrod, 2001; Dunn, 1991), but its effect on particle attachment is not clear.

In the WHO guidelines (WHO, 2006), the recommendation for less than one helminth egg per L of treated wastewater is based on epidemiological evidence. However, dose-response data for *A. lumbricoides* are now available (Navarro et al., 2009), thus it is possible to conduct quantitative microbial risk analysis (QMRA) simulations for *Ascaris* to determine required minimum reduction for restricted and unrestricted irrigation (Mara and Sleigh, 2010). In the process of determining how to achieve the required helminth reduction (sedimentation, filtering, etc.), information such as settling velocities of eggs are crucial for e.g. dimensioning of settling tanks (Chancelier et al., 1998). Until now theoretical calculations of the settling velocities for parasite eggs in water as proposed by Shuval (1978) have been used, whereas a direct determination of the actual settling velocities of helminth eggs remains to be done as has been reported for protozoan parasites (Medema et al., 1998). Additionally, no data exists on the settling mechanism and velocity of helminth eggs in wastewater where some degree of attachment to particles is expected. The aim of this study therefore was to experimentally determine the settling velocity of different helminth egg types in clean water (no particles) and in wastewater (with particles), and to investigate the use of Stokes' law as a predictive model of the settling velocity of helminth eggs in water.

2. Materials and methods

2.1. Recovery of helminth eggs

Eggs of *Ascaris suum*, *Trichuris suis*, and *Oesophagostomum* spp. (Fig. 1) were recovered from feces of naturally infected pigs in Denmark. Eggs were isolated by sieving fresh feces through a series of sieves using cold tap water. The mesh sizes used in declining order were 500, 212, 90 and 38 μm for Ascaris eggs; 500, 212, 90, 38, 35, 31.5 and 30 μm for Trichuris eggs; and 500, 212, 90, and 53 μm for Oesophagostomum eggs. These mesh sizes were used to optimize egg recovery (Jørgensen, 1978; Oksanen et al., 1990). All eggs were then collected on a 20-μm sieve and transferred to a 500 ml glass beaker and left overnight at 5 °C. The following day the liquid upper phase in the beaker was discarded and a number of centrifuge tubes were each filled with 10 ml of the egg suspension left in the beaker. Each tube was resuspended with flotation fluid (50 g glucose monohydrate/100 ml saturated NaCl solution yielding a specific gravity of 1.27 g/ml) to a total volume of 50 ml and subsequently centrifuged for 7 min at 253 *g* (modified from Larsen and Roepstorff, 1999; Roepstorff and Nansen, 1998). The supernatant containing the eggs was drawn off and collected in a new centrifuge tube. The resuspension, centrifugation and collection of the supernatant were repeated three times, and the eggs in the combined supernatant were collected on a 20-μm mesh size sieve and subsequent washed with demineralized cold water. The eggs of Ascaris and Trichuris were removed from the sieve and stored in H_2SO_4 (0.05 M, pH 1, 24 eggs/μl), and Oesophagostomum eggs were stored in demineralized water (24 eggs/μl) at 5 °C (modified from Eriksen, 1990).

2.2. Characteristics of tap and wastewater

The tap water used had the following characteristics: pH, 7.15–7.45; conductivity, 81–168 mS/m; iron (Fe), <0.01–0.09 mg/L; and oxygen (O_2), 7.6–10.7 mg/L (water quality characteristics according to the Frederiksberg Municipality Water Supply). The wastewater used for the sedimentation experiments was obtained from the inlet to the primary settling tanks at the wastewater treatment plant "Lynetten", Copenhagen, Denmark. Characteristics of the wastewater used were as follows: total suspended solids, 240 mg/L; total dissolved solids, 1620 mg/L; pH 7.95; specific density, 0.9987 g/ml at 23 °C. Initial analyses showed that the majority (>87%) of the particles in the wastewater was <20 μm and only a small fraction was larger than 250 μm (results not shown). Examinations of the raw wastewater by sedimentation and flotation showed no occurrence of helminth eggs.

2.3. Sedimentation of helminth eggs in tap and wastewater

Sedimentation experiments in tap water were done with suspensions of 500–600 eggs of each individual parasite

whereas in wastewater the egg suspensions consisted of 500–600 eggs of each parasite together. Sedimentation experiments were done in new Owen tubes (Fig. 2) made of acrylic plastic, and with an internal diameter of 50 millimeters (Owen, 1976). According to Huisman (1995) the small diameter of the Owen tube does not interfere with sedimentation since the upward flow of displaced fluid does not significantly hinder the sedimentation if the particle is smaller than approximately 1/45 of the column diameter. Prior to a sedimentation experiment and after the termination of an experiment, the glass cylinders used for mixing tap and wastewater with parasite eggs as well as the Owen tubes were rinsed well with 0.01% Tween20 solution (Merck, Hohenbrunn, Germany) to minimize egg adhesion to the tube wall, and to remove helminth eggs that adhered to the glass and plastic surfaces to prevent contamination with possible leftovers of eggs to subsequent experiments. Two L of tap water or wastewater kept at room temperature (21–23 °C) were added the suspensions of parasite eggs and then stirred well to secure a homogeneous distribution of eggs and particles, where after the solution was poured into the Owen tubes and allowed to settle. After predetermined time intervals (in tap water: 4, 8, 15, 30, 60, 120, 240, 600, 1320, 2640 min; and in wastewater: 2, 4, 8, 16, 24, 32, 48, 64, 128, 260 min), ten 200-ml subsamples were collected from the bottom of the Owen tubes by a funnel with a clamp into 250-ml glass beakers.

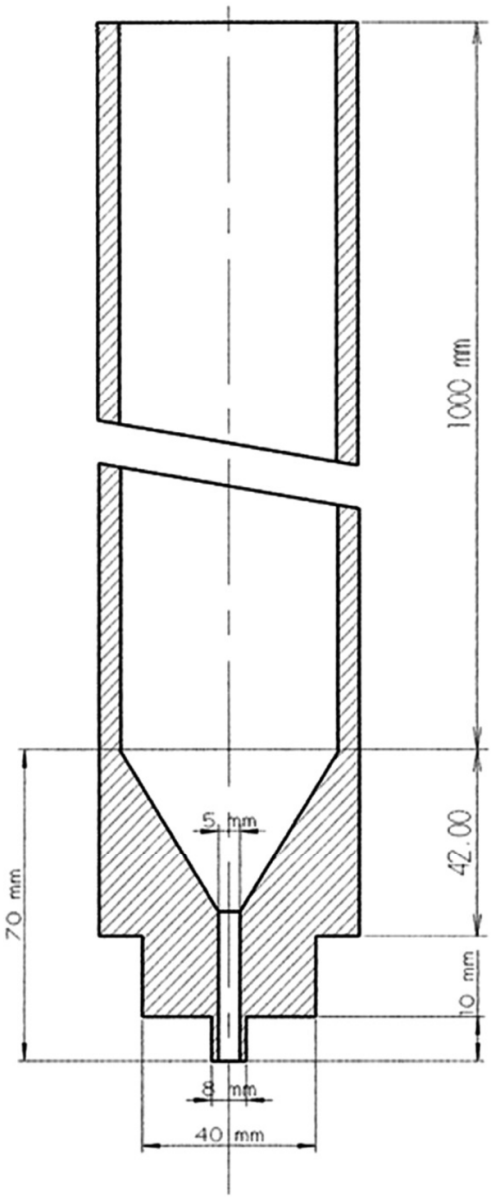

Fig. 2 – Schematic illustration of the sedimentation tube (Owen tube).

Each 200-ml subsample solution was transferred to centrifuge tubes and centrifuged for 7 min at 253 *g*. The supernatant was discarded with a vacuum pump leaving about 10 ml of egg sample suspension in the bottom of each tube. The egg suspensions from these tubes were then transferred into a single tube and each tube was rinsed with 0.01% Tween20 solution to ensure that all eggs were transferred. The combined egg solution was centrifuged and the supernatant discarded leaving 1.5 ml of combined egg suspension. Wastewater samples were further processed to remove particulate matter to ensure that eggs were not trapped in particle flocs, and hence not counted. This was done by centrifuging the single tube containing all the egg suspensions for 7 min at 253 *g* and discarding the supernatant leaving 5 ml of combined egg suspension. Then, 35 ml of 0.01% Tween20 solution was added and the suspension vortexed for 1 min. This solution was then poured through one layer of fine gauze (Cutisoft® Cotton, BSN-Medical, Hamburg, Germany) and the filtrate collected in a glass beaker. The filtrate was transferred back to same tube and centrifuged before the supernatant was discarded as described above. The gauze was rinsed with 40 ml of 0.01% Tween20 in the glass beaker and poured into the centrifuge tube with the remaining 5 ml filtered egg suspension. The sample was centrifuged and the supernatant discarded leaving 1.5 ml of combined egg suspension as was done with the tap water samples. To each combined egg suspension obtained from the wastewater and tap water samples, 6 ml of the above mentioned flotation fluid was added and the entire volume of eggs and flotation fluid transferred with a glass Pasteur pipette to 7–8 McMaster slides. Eggs present both inside and outside the normal gridded areas of the McMaster chamber were enumerated. For each type of helminth egg, the sedimentation experiment was repeated five times in tap water as well as in wastewater.

2.4. *Sedimentation of wastewater particles*

Experiments with wastewater without added helminth eggs were done to determine the sedimentation rate of the particles contained in the water. Wastewater from the same batch used for the egg sedimentation experiments was poured into a 2000 ml glass cylinder, stirred well and then poured into the Owen tube and allowed to settle. After predetermined time intervals (2, 4, 8, 16, 24, 32, 48, 64, 128, 260 min), ten 200-ml subsamples were collected from the bottom of the Owen tubes by a funnel into 250 ml glass beakers. Each subsample was filtered through filters with mesh sizes of 250 μm (polyamide, Sintab Product AB, Oxie, Sweden), 50 μm (polyamide,

Sintab Product AB) and 0.45 μm (cellulose nitrate, Sartorius Stedim Biotech, Aubagne Cedex, France) with a vacuum pump. Prior to filtering the samples, the filters were moistured and dried in an oven for two hours at 60 °C, left on the table for one hour to adjust to temperature and air humidity and weighted with a precision of 0.1 mg. After filtration of the wastewater samples, all the filters were processed and weighted as mentioned above allowing the determination of the dry weight of the particles on the filter. As controls, three sets of filters for each sedimentation experiment were processed with water and handled as described above and used to correct the weight of the filters containing wastewater particles. The sedimentation experiments with particles in wastewater were repeated five times.

2.5. Calculation of settling velocity and statistical analyses

The median settling velocity of eggs and particles in wastewater was calculated using the method described by Owen (Owen, 1976). For eggs, the number of eggs in each sub sample was used in the calculation, and for particles their dry weight in each sub sample was used. The calculation of the settling velocities is based on the difference in egg numbers or particle weights in the ten sub samples obtained from one Owen tube experiment, taken at different time intervals corresponding to a known settling height in the tube (total height 1 m). Thus, one median settling velocity is obtained for each Owen tube experiment. Based on the time interval selected for wastewater sampling (see sections 2.3 and 2.4) from the Owen tubes, the lower limit for estimating the median settling velocity was 0.023 mm s^{-1}. In a few cases, more than 50% of the eggs and particles were settling with a velocity less than 0.023 mm s^{-1} and hence a median settling velocity could not be calculated. In these cases, the settling velocity was set to 0.023 mm s^{-1}. Median settling velocities were log-transformed in order to stabilize variances. One-way ANOVA analyses were performed on the three egg types in tap water and in wastewater separately, to determine if there was a difference in settling velocity between the eggs in each water type. In wastewater, pair-wise comparisons of the settling velocity of each egg type with the particles were performed. Additionally, the settling of each egg type in tap water and wastewater were compared by *T*-tests.

2.6. Theoretical settling velocity of eggs

The settling velocity of particles is anticipated to follow Stokes' law, which is described by the following equation:

$$V_s = g/18 \times d^2 \times \left(\rho_p - \rho_l\right) \times \eta^{-1}$$

where V_s is settling velocity (m s^{-1}), g is gravitational acceleration (9.81 m s^{-2}), d is particle diameter (m), ρ_p is specific density of the particle (kg m^{-3}), ρ_l is specific density of the liquid (kg m^{-3}), and η is dynamic viscosity of the liquid (kg m^{-1} s^{-1}). The parameters in Stokes' equation were determined as follows with regards to egg size, egg density, and liquid density and viscosity.

The egg sizes (d) were determined by measuring the largest diameter (length) and smallest diameter (width) of 100 eggs of each helminth with a Leica microscope using the software program Leica IM500 (version4.0; Leica, Cambridge, United Kingdom). The mean of length and width for each of 100 eggs was used to calculate a mean egg size of each helminth type to be used in Stoke's equation. Confidence intervals (CI) were also calculated. The egg size distribution was also measured with laser particle analyzer (LISST 100C, Sequoia, Seattle, USA) (Agrawal and Pottsmith, 2000) by analyzing the light-diffraction of the egg solutions (0.5–1.5 million eggs in tap water) introduced into the standard 160 × 170 × 190 mm laboratory-chamber of the instrument.

The egg densities (ρ_p) were determined by density gradient centrifugation in a sucrose solution (David and Lindquist, 1982). The following sucrose solutions were used to prepare the gradients: 3% (sp. density 1.0092 g/ml), 13% (1.0551 g/ml), 22% (1.0922 g/ml), 30% (1.1316 g/ml), 35% (1.1535 g/ml), and 54% (1.2516 g/ml). Two ml of each solution was carefully layered using a Pasteur pipette in a 12-ml tube from the lightest (3%) to the heaviest (54%) sucrose solution. Five hundred parasite eggs were layered on top of the gradient and centrifuged at 800 *g* for 20 min and the centrifuge allowed to come to a standstill with the break set to zero. Following centrifugation, each layer in the gradient was carefully transferred to clean McMaster tubes with a Pasteur pipette and suspended in demineralized water to a total of 10 ml. The tube was then centrifuged at 253 *g* for 7 min and the supernatant removed with a vacuum pump leaving 0.5 ml suspension. To each egg suspension, 4 ml of the above mentioned flotation fluid was added and the total number of eggs in the entire volume was counted in McMaster chambers as described earlier. Egg specific density was calculated as weighted mean, and the mean of three determinations for each helminth egg type was used in Stokes' equation.

The specific density (ρ_l) of tap water and wastewater were measured at 23 °C with a glass pycnometer. The mean of three measurements was used in Stokes' law. The kinematic viscosity of tap water (η) was determined at 23 °C by a calibrated viscometer with a viscosity range of 0.9 to 3 mm^2 s^{-1}. The dynamic viscosity (η) was calculated from the kinematic viscosity (υ) by the following equation:

$$\eta = \upsilon\rho$$

where η is dynamic viscosity (kg m^{-1} s^{-1}), υ is kinematic viscosity (m^2 s^{-1}), and ρ is density of the medium (kg m^{-3}). The mean of three viscosity determinations was used in Stokes' equation.

3. Results

3.1. Settling velocity of helminth eggs in tap water and wastewater

The results of the sedimentation experiments in tap water are shown in Fig. 3A. The mean settling velocity calculated based on the outcome of the five determinations for each egg type, was 0.0612 ± 0.0053 mm s^{-1} (±SD) for Ascaris eggs, 0.1487 ± 0.0602 mm s^{-1} for Trichuris eggs, and 0.1262 ± 0.0219 mm s^{-1} for Oesophagostomum eggs. The settling velocity of Ascaris was significant lower ($P < 0.05$) than the velocity of the

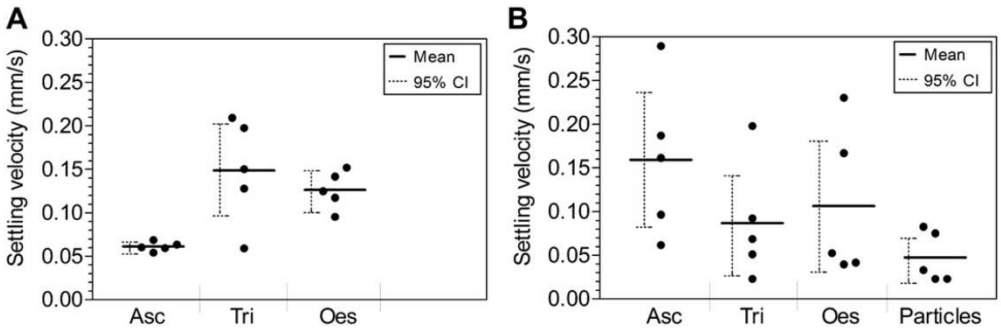

Fig. 3 – Settling velocity in five experiments carried out with Ascaris (Asc), Trichuris (Tri) and Oesophagostomum (Oes) in tap water (A) and in wastewater, including natural occurring particles (B).

two other helminth egg types. There was very little variation in the five experimental repeats for Ascaris compared to the data for Trichuris and Oesophagostomum. The overall recovery of the three helminth eggs was 70–80% in tap water.

The results of the sedimentation experiments with helminth eggs in wastewater are shown in Fig. 3B. The mean settling velocity calculated based on results obtained from the five determinations for each egg type, was 0.1593 ± 0.0883 mm s^{-1} for Ascaris eggs, 0.0866 ± 0.0672 mm s^{-1} for Trichuris eggs, and 0.1062 ± 0.0875 mm s^{-1} for eggs of Oesophagostomum. There was significant difference between the settling velocities of Ascaris and the two other helminth egg types in wastewater ($P < 0.05$). The wastewater particles had a mean settling velocity of 0.0474 ± 0.0292 mm s^{-1} which was significantly different from the settling velocity of Ascaris eggs ($P < 0.05$), but not significantly different from Trichuris and Oesophagostomum.

Ascaris eggs had a significantly higher ($P < 0.05$) settling velocity compared to tap water. In contrast, Trichuris and Oesophagostomum eggs seemed to have decreased settling velocities in wastewater compared to tap water. The recovery of helminth eggs in wastewater was generally lower than in tap water: 57–80%, 54–59%, and 27–47% for Ascaris, Trichuris and Oesophagostomum, respectively.

3.2. *Theoretical settling velocity*

To allow for a comparison of the experimental and theoretical (Stokes' equation) settling velocities, the values of the equation parameters (egg size and density; liquid density and viscosity; and settling velocity) and their variations were determined for the conditions used in the experiments.

The measured eggs sizes (width, length and mean diameter) together with the mean eccentricity (ratio between length and width) are listed in Table 1. Overall, Trichuris had the smallest eggs and Oesophagostomum the largest sized eggs. None of the eggs were spherical (eccentricity of 1) which is assumed by Stokes' law for calculating the settling velocity, nevertheless Ascaris eggs were more spherical compared to eggs of Trichuris and Oesophagostomum (Fig. 4). Since none of the eggs are spherical, it is likely that they settle with different but unknown orientations due to Brownian motions in the Owen sedimentation tube. Thus, the mean of length and width of the eggs was used in Stoke's equation as an approximation of the dynamic diameter of helminth eggs. The mean size used for Ascaris eggs was 61.31 ± 3.58 μm, for Trichuris eggs 46.47 ± 2.03 μm, and for Oesophagostomum eggs 63.25 ± 1.11 μm. A random orientation of the eggs was reflected in the egg size distribution, as measured by the LISST laser particle analyzer (Fig. 5), showing a very narrow peak for close-to-spherical Ascaris eggs and a wider distribution of Oesophagostomum and Trichuris with ellipsoid eggs.

Densities for Ascaris, Trichuris and Oesophagostomum eggs were 1.12 ± 0.007 kg m^{-3}, 1.10 ± 0.010 kg m^{-3}, and 1.07 ± 0.002 kg m^{-3}, respectively. In tap water, the mean specific density was 0.9978 ± 0.0011 kg m^{-3}; the mean kinematic viscosity was 0.9424 ± 0.0068 mm^2 s^{-1} and the dynamic viscosity was calculated as 0.000940 Pa s.

The theoretical settling velocity of the helminth eggs was calculated using the above mentioned equation parameters in Stokes' law, and is shown in Table 2. The theoretical settling velocity for Trichuris (95% CI, 0.1270–0.1314) shows overlap in confidence intervals with the experimentally observed velocity (95% CI, 0.0959–0.2015). However, for Ascaris the theoretical settling velocity (95% CI, 0.2686–0.2813) was three times higher than the observed settling velocity (95% CI, 0.0565–0.0658) and for Oesophagostomum the theoretical

Table 1 – Measured sizes of helminth eggs used in the experiments.

Parameter	Egg size (μm)		
	Mean (±SD)	Min	Max
Ascaris			
Length	67.20 (5.33)	52.16	84.07
Width	55.41 (3.91)	46.80	64.64
Eccentricity	1.22 (0.12)	1.00	1.80
Trichuris			
Length	62.16 (2.60)	54.25	68.02
Width	30.78 (2.72)	26.67	38.29
Eccentricity	2.03 (0.16)	1.56	2.38
Oesophagostomum			
Length	76.17 (5.52)	62.74	85.67
Width	50.33 (6.63)	38.35	60.23
Eccentricity	1.53 (0.15)	1.24	1.99

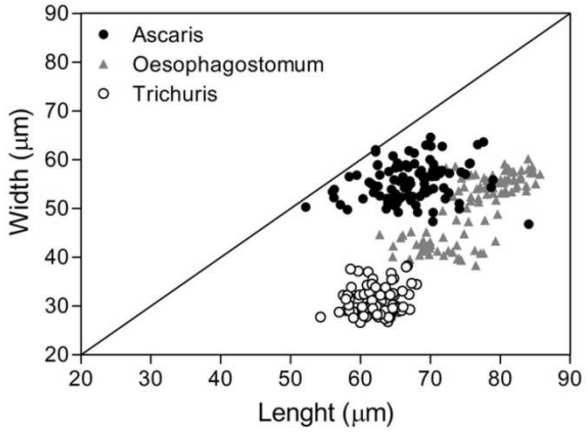

Fig. 4 – Egg size distribution of helminth eggs. The solid line represents an eccentricity of 1 (ratio between length and width).

(95% CI, 0.1504–0.1613) was only slightly higher than the experimental settling velocity (95% CI, 0.1069–0.1454).

4. Discussion

The results of the sedimentation experiments in tap water showed that Ascaris eggs had the slowest settling velocity compared to the settling velocities observed for Trichuris and Oesophagostomum eggs. Our findings are different when compared with the theoretical calculations made by Shuval (1978) for human helminth eggs which estimated that Trichuris eggs (0.425 mm s^{-1}) settle the fastest, followed by Ascaris eggs (0.181 mm s^{-1}) with hook worm eggs (0.108 mm s^{-1}) settling with the slowest velocity. As also documented in our study, Stokes' law (see formula above) has some assumptions and limitations (Gordon et al., 2004). A falling particle is assumed to be spherical and have a smooth surface, which is generally not the case for helminth eggs (see Fig. 1 and Table 1). Despite the non-spherical shape of

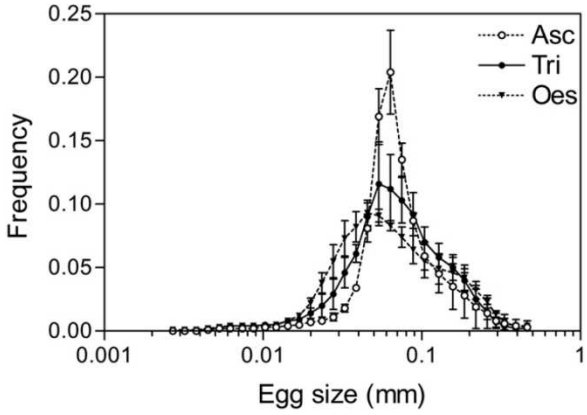

Fig. 5 – The egg size distribution measured with LISST 100C laser particle analyzer. Note the log-scale on X-axis.

helminth eggs, our predicted settling velocities for Trichuris and Oesophagostomum eggs showed good agreement with the observed velocities found in the Owen tube experiments (Table 2). For Ascaris eggs, the theoretical settling velocity was much higher than the observed. This could be explained by the fact that the surface of Ascaris eggs are mammillated and not smooth as is the surface of the other two eggs (Fig. 1). Stokes' law is only valid for laminar flow (fluid layers gliding over one another) which is seen only for particles with smooth surfaces. Thus, the mammillated surface structure of Ascaris eggs may cause a turbulent flow so the fluid layers move in random irregular paths (Gordon et al., 2004) which decrease the settling velocity. Other studies (e.g. Sosnoskie et al., 2009) support our findings that a turbulent flow around particles will lead to an overestimation of settling velocity using Stokes' law.

Variations in egg density and egg size have a major impact on settling velocity as calculated by Stokes' law. In our study, the settling velocities of the different egg types was calculated using measured values of egg density, egg size and liquid density and viscosity. Settling velocities obtained were 0.2749 mm s^{-1} for Ascaris, 0.1292 mm s^{-1} for Trichuris and 0.1581 mm s^{-1} for Oesophagostomum (Table 2). These settling velocities are higher for Ascaris and Oesophagostomum eggs and lower for Trichuris eggs than the settling velocities calculated by Shuval (1978) who used density and size information on the corresponding human parasites (Faust et al., 1970). The egg densities used by Shuval (1978) were similar to the densities measured in this study, only Shuval (1978) found that Trichuris eggs had higher density (1.15 kg m^{-3}). David and Lindquist (1982) reported egg densities of the three helminth egg types collected from pigs and dogs (*Ancylostoma caninum*) that were similar to our findings, but found a higher density of Trichuris (1.13 kg m^{-3}) eggs. It is unknown if there exist host-dependent variations in density of different helminth egg types. However, it is documented that helminth eggs in the environment exhibit individual density variations depending on age or stage of development, e.g. degree of embryonation (Magat et al., 1972; Sawitz, 1942). We were surprised to find a lower mean density for Trichuris than Ascaris eggs, as it is widely recognized that the former is more difficult to detect and count in the laboratory using standard flotation techniques. The difficulties in floating Trichuris eggs may be associated with a much larger variation in egg density as compared to Ascaris and Oesophagostomum eggs as suggested by our findings that a fraction of 5–10% showed a density above 1.25 kg m^{-3} (data not shown). If a proportion of the eggs do not float in standard flotation fluids, e.g. saturated salt solutions, this will inevitably lead to an underestimation of egg numbers. The egg sizes that Shuval (1978) used (*A. lumbricoides*, 55 × 40 μm; *T. trichiura*, 50 × 22 μm; hookworm 60 × 40 μm) for calculating settling velocities are considerably smaller than the sizes measured in this study which was based on 100 measurements of individual eggs of Ascaris, Trichuris and Oesophagostomum (Table 1). In our calculations of settling velocities we used the mean of length and width of the eggs as an approximation of the dynamic diameter of the eggs. Shuval (1978) in contrast did not describe which egg diameters he used in Stokes' equation. The differences in egg densities, especially for Trichuris eggs, and sizes for all three egg types, result in marked

Table 2 – Experimental and theoretical (using Stokes' law) mean settling velocities for helminth eggs in tap water.

	Experimental settling velocity (mm s^{-1}) [m h^{-1}]		Theoretical settling velocity (mm s^{-1}) [m h^{-1}]	
	Mean	95% CI	Mean	95% CI
Ascaris	0.0612 [0.22]	0.0565–0.0658	0.2749 [0.99]	0.2686–0.2813
Trichuris	0.1487 [0.54]	0.0959–0.2015	0.1292 [0.47]	0.1270–0.1314
Oesophagostomum	0.1262 [0.45]	0.1069–0.1454	0.1581 [0.57]	0.1504–0.1613

differences in egg settling velocities as demonstrated in this study and by Shuval (1978).

Our Owen tube sedimentation experiments showed that Trichuris and Oesophagostomum eggs had decreased settling velocities in wastewater, whereas the velocity of Ascaris eggs increased compared to the settling velocities found for eggs in tap water (Fig. 3). Trichuris and Oesophagostomum eggs were found to have similar mean settling velocity as the particles in the wastewater where as Ascaris eggs settled faster. It should be noted that the range of velocities of the eggs where broader than for the particles. The decreased settling velocity of Trichuris and Oesophagostomum eggs is likely due to entrapment of eggs to the flocculated particles. The entrapment of eggs may also explain the lower egg recovery in wastewater than in tap water. Unfortunately, it was impossible to determine the size distribution of the particle flocs with the LISST particle analyzer due to high water turbidity. The very low settling velocity measured for flocculated particles in wastewater as compared to settling velocities reported in saline water indicates that the water content of the wastewater flocs was very high (Gibbs, 1983). The similar settling velocity of wastewater particles and Trichuris and Oesophagostomum eggs therefore strongly suggests that the eggs were incorporated into flocs with low density. This means that the behavior and settling velocity of the particles in wastewater determines the settling velocity of these two helminth egg types. It is generally anticipated that particle flocs settle faster than individual suspended particles (Kim and Stolzenbach, 2004). The increased settling velocity of Ascaris eggs is most likely due to their adhesion to particles which then increase the floc size and hence enhances the settling velocity. We can only speculate on the reason for the different behavior of these eggs, but it could be that the mammilated surface of the Ascaris egg (and the higher density of these eggs) causes entrapment in other types of particle flocs that settle faster than the smooth eggs of Trichuris and Oesophagostomum. This particular aspect could be the subject for future studies on settling of Ascaris eggs. Helminth eggs from Trichuris and Oesophagostomum entrapped in flocs settled slower compared to eggs in tap water. It should be noted that Stokes' law assumes that eggs settle in isolation (Gordon et al., 2004) which seems to only occur in clean water. Thus, because of the observed interaction between eggs and other particles, our study demonstrates that Stokes law is only suitable for calculating settling velocities of helminth eggs in clean water types, e.g. drinking water, and not in low quality water like wastewater. Aquatic recipients contaminated with wastewater containing helminth eggs, e.g. rivers used as sources of irrigation water, and waste stabilization ponds do also often have a high concentration of suspended particles resulting in flocculation of the suspended material, including the eggs. It is therefore likely that helminth eggs in different types of surface waters do not settle as single entities but rather as part of flocs consisting of fine-grained suspended material (Droppo, 2001). Future studies should assess how different types of wastewater (e.g. treated/un-treated, composition of organic materials, particle concentration and particle size) influence the flocculation and settling of helminth eggs. Such studies should also consider the water salinity as it is well-documented that flocculation increases significantly in saline waters (Gibbs, 1983). Changes in concentration of suspended material are expected to increase settling velocity with increasing concentration of such material leading to more flocculation (van Leussen, 1999). Variations in other particle parameters can also be expected to significantly influence flocculation in complex, but poorly understood associations (Droppo, 2004).

Our observed low settling velocities of the flocculated suspensions, including eggs, in low quality water are unlikely to result in any significant sedimentation in most aquatic habitats where turbulence events caused by water flow, wind, rain, temperature, and human disturbances seem to be more important than gravitational settling in determining the sedimentation of eggs in water. When low quality water, e.g. treated wastewater, is assessed for its safe use for irrigation of crops, Ascaris is usually used as the main indicator organism due to the long survival time of the eggs and low infectious dosage compared with other parasite, bacterial and viral pathogens (Feachem et al., 1983). As the major factor determining helminth egg removal is sedimentation, the efficiency of different aquatic environments, including wastewater treatment systems, in removing eggs should be based on the helminth egg type with the lowest settling velocity. This study clearly documents that in clean water Ascaris eggs should be used as an indicator of fecal pollution with helminth eggs due to its slow settling velocity (Fig. 3A), whereas in low quality water like wastewater Ascaris eggs settle with the fastest velocity and this egg type is therefore not a good indicator of the removal rate of other helminth types by sedimentation (Fig. 3B). Trichuris and Oesophagostomum eggs settle slower and there is a need to differentiate the type of helminth eggs when assessing the quality of low quality water, e.g. when implementing the WHO guidelines of less than one helminth egg per liter (WHO, 2006). Our findings that Trichuris and Oesophagostomum eggs settle with the same velocity as wastewater particles indicates that measurements of particle settling velocity in treated low quality water, e.g. in waste stabilization ponds, may be used as an indicator of helminth egg settling velocity. Further studies are needed to document how these settling velocities may be used to model helminth

egg removal. It is also proposed that existing models of helminth egg removal, such as the model of Ayres et al. (1992), incorporate our findings to refine the models.

5. Conclusion

Our study documents that not all helminth eggs settle in clean water according to Stokes' law which sometimes overestimates the settling velocity. This must be taken into consideration when Stokes' law is used to model the helminth egg removal in water, e.g. when designing water treatment facilities. Furthermore, the settling velocity and behavior of Trichuris and Oesophagostomum eggs in water is determined by the presence of particles in water, whereas Ascaris eggs show a faster settling velocity. Therefore further studies are needed to assess how different types of particles and concentrations of suspended materials in different types of low quality water influence the flocculation and settling of helminth eggs. Additionally, at which concentration of suspended materials (ranging from clean water to wastewater) Stokes' law becomes inadequate as a predictive model must be explored. A main implication of our findings is that there is a need to differentiate between the settling velocities of different helminth egg types when assessing the safety of low quality water, e.g. for use in irrigation.

Acknowledgements

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4.2 Paper II

Resuspension and settling of helminth eggs in water: interactions with cohesive sediments

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Resuspension and settling of helminth eggs in water: interactions with cohesive sediments

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Abstract

Helminth parasite eggs in low quality water represent main food safety and health hazards and are therefore important indicators used to determine whether such water can be used for irrigation. When helminth eggs are removed from water by sedimentation they accumulate in the sediment. Resuspension of deposited helminth eggs in e.g. irrigation canals will lead to increased concentration of suspended eggs in the water. Our study aimed to determine the erodibility (erosion rate and erosion threshold) and settling velocity of *Ascaris* and *Trichuris* eggs as well as cohesive sediment at different time points after incorporation into the sediment. Cohesive sediment collected from a freshwater stream was used to prepare a sediment bed onto which helminth eggs were allowed to settle. The erodibility of both sediment and helminth eggs was found to decrease over time indicating that sediment consolidation takes place along with incorporation of eggs into the sediment. This was supported by finding of a higher settling velocity for eggs associated with particles as compared to eggs in clean water. The incorporation into the sediment bed decrease the mobility of helminth eggs and the aggregation increases the effective settling velocity of the eggs leading to reduced mobility. Our findings documents that helminth eggs should not be viewed as single entities in water systems when modeling the distribution of eggs since both erodibility and settling velocity of eggs are determined by mobility of the sediment present in the water stream. Recalculation of the erosion threshold for helminth eggs and sediment showed that even at relatively low current velocities i.e. 0.07-0.12 m s^{-1} the eggs will demonstrate high mobility in open

irrigation channels. These environmental factors affecting resuspension must be taken into account when developing models for sedimentation of helminth eggs in different water systems.

Keywords: resuspension, erosion, helminth eggs, aggregation, particles, settling velocity

1. Introduction

Freshwater sources are becoming increasingly scarce in many parts of the world, and use of wastewater has become an attractive option for conserving and expanding available water supplies. Wastewater use in agriculture has a long history and it has been estimated that about 20 million hectares of agricultural land worldwide is irrigated with treated or untreated wastewater (Jimenez and Asano, 2008). In low-income countries, the sources of irrigation water are water bodies, i.e. streams, shallow ponds and drains, which often are contaminated with wastewater due to lack of proper treatment facilities (WHO and UNICEF, 2000). Especially when untreated wastewater is used for crop irrigation, it poses substantial risks to human health, not only for farmers, but also for surrounding communities and consumers of the crops, in particular when crops are eaten uncooked (Blumenthal *et al.*, 2000). The major health risks from irrigation with wastewater are associated with viral, bacterial, and parasite pathogens that are usually present in untreated, partially treated, and occasionally treated wastewater (Feachem *et al.*, 1983; Shuval *et al.*, 1986).

Helminth parasite eggs in wastewater are of particular health concern due to the high burden of helminth diseases in low-income and lower-middle-income countries where use of wastewater is most prominent (WHO, 2006). The most important intestinal helminths are *Ascaris lumbricoides* (the human roundworm), *Trichuris trichiura* (the human whipworm) and *Ancylostoma duodenale* and *Necator americanus* (the two human hookworms) (Feachem *et al.*, 1983; Shuval *et al.*, 1986). It is estimated that at least 1.2 billion people globally are infected with one or more of the four species of intestinal worms (Bethony *et al.*, 2006; de Silva *et al.*, 2003). The helminth eggs are shed by infected humans within whom the female worms produce vast quantities of eggs (up to 200,000 per day) (Feachem *et al.*, 1983). The eggs enter the wastewater through direct faecal input, discharge of treated and untreated sewage, and surface water runoff from agricultural lands (Kirby *et al.*, 2003). The occurrence and concentration of helminth eggs in wastewater depend on a variety of factors, i.e. the helminth species, the number of infected persons serving as the source, and the volume and concentration of sewage (Shuval *et al.*, 1986). The concentration of *Ascaris* in raw wastewater may

vary from 10-100 eggs per L in endemic areas to 100-1000 eggs per L in hyperendemic areas (Kamizoulis, 2008; Mara and Sleigh, 2010). In most wastewater treatments (from conventional to simple waste stabilization ponds) and in contaminated ponds or slow-flowing streams helminth eggs are removed by sedimentation processes (Scheierling *et al.*, 2010). Since helminth eggs are highly resistant to environmental stress and remain viable for several months and even years (Feachem *et al.*, 1983), a high degree of egg removal is required by treatment if wastewater is to be used safely in irrigated agriculture (Stott, 2003). In its recent guidelines the World Health Organization (WHO) recommends that wastewater to be used for irrigation should contain less than one helminth egg per litre when no other risk reduction options are available (WHO, 2006). When helminth eggs are removed from the water by sedimentation they are accumulated in sludge and sediment which represent a health hazard when the sludge is handled and used as crop fertilizer (Nelson, 2003). Further, resuspension of deposited helminth eggs from sediments in e.g. irrigation canals or in natural ponds will lead to increased concentration of suspended eggs in the water. However, no data exist on the resuspension of settled helminth eggs in aquatic environments.

Cohesive sediment, or mud (here defined as particles with a grain size <63µm), is a mixture of clay particles, silt, organic material, and in general a large amount of water. This sediment has cohesive properties because of the electrochemical attraction of clay particles and organic material and will therefore form aggregates (Eisma, 1986). Direct or indirect aggregation may also take place due to presence of microphytobenthos (Paterson, 1989; Yallop *et al.*, 1994) and bacteria (Grant and Gust, 1987). Macro-fauna will tend to aggregate cohesive sediment through feeding or movement activities (Andersen, 2001; de Brouwer *et al.*, 2000; de Deckere *et al.*, 2000). The erodibility or erosion potential (defined as the ease with which particles are removed from the bed surface and put into suspension again) of cohesive sediment beds will typically show strong dependence on these physical and biological factors and processes, i.e. a dependence on grain size composition (e.g. mud content), bed roughness and biological activity (Black *et al.*, 2002).

Sediment cohesion and aggregation has a marked effect on both the erodibility and settling velocity of the sediment, typically resulting in increased erosion resistance of sediment beds and increased settling velocity of suspended sediment (Andersen and Pejrup, 2002; Nowell *et al.*, 1981). Both physical and biological properties of sediments may display seasonal variation, and hence temporally varying erodibility and settling velocity (Andersen, 2001). Sediment particles deposited

on fine-grained beds in nature are incorporated into the sediment bed over time due to consolidation and the range of biological and physio-chemical processes mentioned above. If and to what extent helminth eggs take part in the aggregation of fine-grained particles in nature is unknown. The eggs of *Ascaris* are widely quoted to be sticky and adhere to surfaces of e.g. vegetables (Gaspard *et al.*, 1994; Jimenez, 2007; Raisanen *et al.*, 1985). *Ascaris* eggs were found to have a negative surface charge in water, but its effect on particle adhesion is not clear (Dunn, 1991). The adherence of helminth eggs to surfaces is also addressed in the WHO manual for analysis of helminth eggs in wastewater which recommends rinsing of the plastic containers used to store the wastewater with detergent to release any adhering helminth eggs (Ayres and Mara, 1996). In a recent study by Sengupta *et al.* (2011), *Trichuris* eggs were found to have similar settling velocity as the particles in wastewater suggesting that the eggs were incorporated into particle flocs, whereas *Ascaris* eggs settled faster than the wastewater particles. This finding may have implications for resuspension of helminth eggs since egg-entrapment in settling particle flocs may cause better incorporation into the sediment bed. However, the magnitude of incorporation of eggs may also depend on the sediment composition and incorporation time.

The aim of this study was to investigate the resuspension characteristics (erosion rate and erosion threshold) and settling characteristics (settling velocity) of helminth eggs and cohesive sediments in natural freshwater. The erodibility of two helminth egg types and the bulk sediment was compared at different time points after incorporation into the sediment. Additionally, settling velocity of the resuspended eggs and sediment was determined at the same time points to study the effect of aggregation between helminth eggs and particles.

2. Material and methods

2.1 Bed preparation and experimental design

Cohesive sediment was collected in July 2011 from Tibberup freshwater stream (55°45'10"N; 12°25'23"E) which is a two m wide and one m deep creek draining the marsh ‘Smørmose’. The sediment was wet-sieved through a 0.5 mm mesh in order to retain macro-fauna and partly decomposed plant material. Eight hundred ml of the sieved sediment suspension was placed in each of nine tubes closed at the bottom; one tube for each erosion experiment (tube dimensions are described in section 2.2). Nine hundred ml of stream water was added yielding a total volume of 1700 ml and the material was allowed to settle for 20 hours creating a sediment bed.

Eggs of *Ascaris suum* and *Trichuris suis* were recovered from feces of naturally infected Danish pigs by sieving and flotation (Sengupta *et al.*, 2011). The pig helminths, *A. suum* and *T. suis* are often used as model organisms for the corresponding human helminth parasites, *A. lumbricoides* and *T. trichiura* (Boes and Helwigh, 2000). The eggs of pig helminths are virtually identical in morphology and size to the corresponding human parasite eggs and are relative easy to obtain in high numbers from infected pigs. An amount of 20,000 eggs (11,765 eggs per litre) of each helminth species (*Ascaris* and *Trichuris*) was added to each of the nine tubes and allowed to settle and deposit on the bed. Erosion experiments were carried out on days one, two and three after bed preparation with three replicates each day, giving a total of nine erosion experiments. The sediment cores were kept at 20-22 °C and exposed to normal daylight in the laboratory (no direct sunlight).

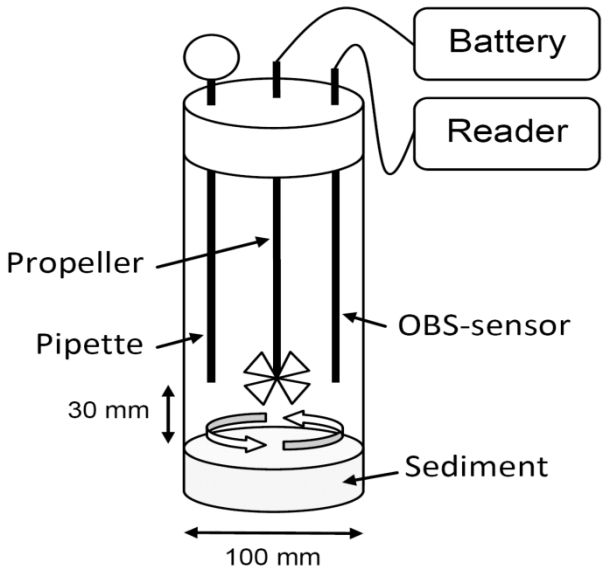

Figure 1. Schematic illustration of the erosion device used for determination of erosion rates and erosion threshold.

2.2 Erosion experiments

Erosion threshold and erosion rate were determined using a portable version of the German EROMES erosion equipment (Andersen, 2001; Schünemann and Kühl, 1991). The device consists of an acrylic tube with an internal diameter of 10 cm and a height of 22 cm (Fig. 1). Bed shear stress was generated by a propeller induced flow and the suspended particulate matter concentration (SPMC) was monitored by an optical back-scattering (OBS) sensor. The propeller revolutions were converted to bed shear stress by use of a calibration based on erosion of quartz sand with known critical erosion shear stress and additional measurements with a hot-film probe (Andersen, 2001). Based on examinations of the Reynolds number during the calibration with quartz sand it was found that a partially smooth flow was present for bed shear stress below 0.5 N m^{-2}, whereas the flow was rough and turbulent for higher bed shear stresses. The bed shear stress was gradually increased 5 times, every six minutes (0, 6, 12, 18, 24, 30, and 36 min) from 0 to 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 N m^{-2}. The OBS readings where

registered every 30 sec and calibrated by SPMC measurements. For the calibration, the SPMC in two 10-ml samples collected with a pipette prior to every increase in bed shear stress during the erosion experiments were measured. The number of helminth eggs was enumerated in three 10-ml samples collected in the same way and at the same time as the SPMC samples.

2.3 Settling experiments

Aggregation and settling velocity of the suspended material and the helminth eggs were analyzed as part of the erosion experiments. The change in SPMC and egg numbers was monitored after the propeller was turned off at the maximum bed shear stress of 0.5 N m^{-2} used in this study and the suspended material and eggs were allowed to settle for six minutes. The OBS readings were registered every 10 seconds during first three min, and every 30 sec in the last three min. The number of helminth eggs was counted in samples of 10 ml collected with a pipette every minute. After the six min of settling two 10-ml samples for SPMC determination and OBS calibration, and three 10-ml samples for egg enumeration were taken. Additionally, the settling velocity of helminth eggs in clean tap water was determined in five separate experiments as controls with the same apparatus and method as mentioned above but without the presence of sediment.

2.4 Determination of SPMC and number of helminth eggs

The SPMC was measured by filtering the pooled samples (2 × 10 ml) through 0.45 µm cellulose nitrate filters (Sartorius Stedim Biotech, Aubagne Cedex, France) with a vacuum pump. Prior to filtering the samples, all the filters were moistured and dried in an oven for two hours at 60 °C, left on the table for one hour to adjust to temperature and air humidity and weighted with a precision of 0.1 mg. As controls, three sets of filters for each oven tray were processed with water and handled as described above and used to correct the weight of the filters containing suspended sediment. The number of helminth eggs was counted using a modified McMaster technique (Sengupta *et al.*, 2011) in the three pooled samples obtained prior to every increase in bed shear stress during erosion experiments.

2.5 Sediment properties

Sediment samples from the top 3 to 5 mm of each core was taken with a pipette and analyzed for various properties characterizing the sediment. For the physico-chemical properties, the grain size distribution of the sediment was determined by laser-sizing using a Malvern Mastersizer 2000

(Malvern Instruments Ltd, Worcestershire, UK) after addition of 0.01 M $Na_2P_4O_7$ and sonification for 2 min with a Branson Sonifier 250 (Emerson Electric Co., St. Louis, USA). Water content was determined after drying at 105 °C, and the dry bulk density was estimated on the basis of the water content using the formulations given by Flemming and Delafontaine (2000). The organic content was determined by loss on ignition (LOI) for 2 h at 550 °C (DIN 18128). The biological sediment properties included the determination of chlorophyll *a* and its degradation product pheopigment spectrophotometrically after extraction in 90% acetone for 24 h at 5°C in darkness. After centrifugation at 250 *g* for 15 min, the absorbance of the supernatant at 665 and 750 nm was measured before and after acidification with 200 µl of 1 M HCl and calculations of the chlorophyll *a* and pheopigment content were based on Parsons *et al.*(1984). The chlorophyll *a* content of the surface layer was used as an indicator of the amount of living diatoms. For bacterial cells, triplicate samples of 1-2 g wet sediment from each sediment core were fixated in 4% formalin for 24 hours. Each sample was homogenized (Braun GmbH, Kronberg/Taunus, Germany) at full speed in 230 ml sterile filtered Mili-Q water for 3 × 1 min, diluted with Mili-Q water, filtered through 0.2 µm polycarbonate membranes that were prestained black (Nucleopore, Pleasanton, California) and then stained for 2 min in 1:10,000 acridine orange (Hobbie *et al.*, 1977). The bacterial cells were separated into three size groups: 0.35-0.70 µm (round, small), 0.71-1.4 µm (round, large), and >1.4 µm (elongate). At least 100 bacterial cells were directly counted in each sample using fluorescence microscopy.

2.6 Calculations and statistical analysis

The egg numbers in suspension and SPMC at each bed shear stress were used to calculate erosion rates based on the volume of water, the sediment bed area and length of the time-step. The erosion threshold of the particles and helminth eggs for each of the three days (for eggs, only day 2 and 3) was determined by plotting the calculated erosion rate as a function of the bed shear stress. By making a linear fit it is possible to determine the threshold (as bed shear stress) at the intercept of a line with a critical erosion rate, in this case 0 g m^{-2} s^{-1} (particles) and 0 eggs m^{-2} s^{-1} (eggs). The critical erosion rate was set for determining the bed shear stress at which a measureable erosion rate is found, i.e. at the earliest erosion observed.

In the erosion experiments, the responses of suspended particles (log-transformed) and particle erosion rates were analysed by an ANOVA with time (shear stress) as repeated measures and the

full factorial design of the fixed effects: day (1, 2, and 3) and suspended particles at time zero as a covariate. Time (0, 6, 12, 18, 24, 30, and 36 min) corresponds to the applied bed shear stress (0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 N m^{-2}) and was used in the statistical analyses since the stepwise increase is the same, whereas in shear stress the increase is smaller in the first three steps. The responses of suspended helminth eggs (log-transformed) and egg erosion rates were analysed in a similar way by an ANOVA with time (shear stress) as repeated measures and the full factorial design of the fixed effects: day (2 and 3), egg type (*Ascaris*, *Trichuris*) and suspended eggs at time zero as a covariate. In the settling velocity experiment, three-way ANOVA was performed with the fixed effects: element type (sediment/*Ascaris* eggs/*Trichuris* eggs), day (1, 2, and 3) and water type (tap water/stream water). For the sediment properties, one-way ANOVA analyses were performed with day (1, 2, and 3) as a fixed effect. For all ANOVA analyses the non-significant effects were removed by backward model reduction on a 5% significance level.

Table 1. Sediment properties in relation to time after establishment of the sediment bed (means±SD).

Sediment property	Day 1[a]	Day 2[a]	Day 3[a]
Dry bulk density (g cm^{-3})	0.89 (±0.01)	0.88 (±0.01)	0.88 (±0.01)
Organic content, LOI (%)	12.3 (±0.1)	12.5 (±1.0)	12.1 (±0.2)
Chlorophyll *a* (µg g^{-1} sediment)	10.01 (±6.36)	12.32 (±3.63)	10.54 (±3.44)
Pheopigment (µg g^{-1} sediment)	5.54 (±5.48)	7.02 (±3.71)	5.02 (±2.33)
Bacterial cells (cells × 10^8 g^{-1} sediment)			
- Round, small (0.35-0.70 µm)	5.8 (±1.5)	5.6 (±4.3)	3.8 (±1.6)
- Round, large (0.71-1.40 µm)	5.4 (±2.4)	3.9 (±1.8)	3.5 (±1.9)
- Elongate (>1.4 µm)	2.4 (±0.8)	1.2 (±0.6)	1.4 (±1.3)

[a] No significant differences between columns.

3. Results

3.1 Sediment properties

Table 1 shows the results of the different sediment properties important for sediment stability. For all the properties, no significant differences were observed within the three cores each day and between the three days. Figure 2 shows the primary grain size distribution of the sediment. It was observed that particle aggregates increased in size over the three days, although this was not measured.

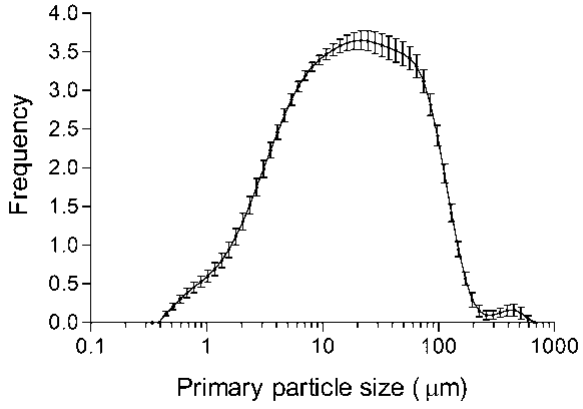

Figure 2. The primary particle size distribution of the sediment used in the erosion experiments. Mean ± SD.

3.2 Resuspension, erosion rate and erosion threshold

The resuspension results of *Ascaris* and *Trichuris* eggs for day 1 were discarded since the eggs had not settled properly before starting the erosion experiments. The results of the erosion experiments are given in Figure 3 and shows that an increase in bed shear stress resulted in a highly significant increase in the amount of both suspended particulate matter and eggs (P<0.0001). The effect of day was significant for the number of suspended helminth eggs with more eggs in suspension on day 2

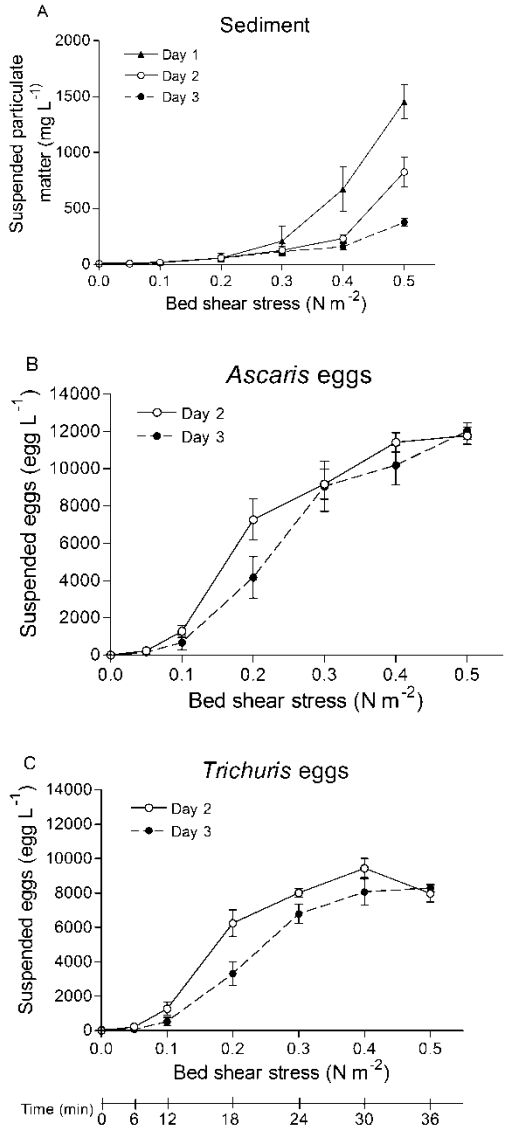

Figure 3. Concentration of suspended sediment (A), *Ascaris* eggs (B) and *Trichuris* eggs (C) in the water on two or three consecutive days. Mean ± SEM. Note the time scale (min) in the bottom of the figure which corresponds to the applied bed shear stress.

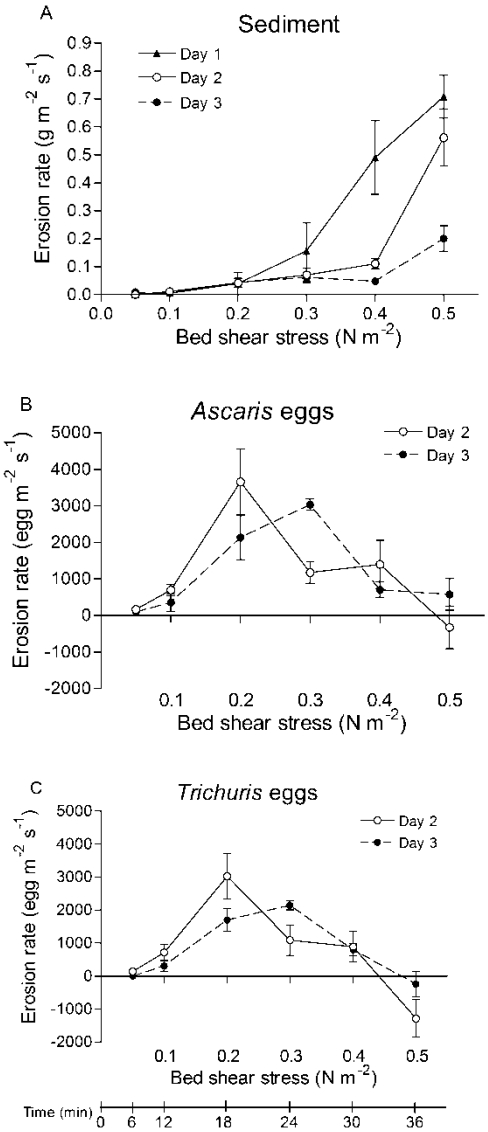

Figure 4. The erosion rate of sediment (A), *Ascaris* eggs (B) and *Trichuris* eggs (C) on two or three consecutive days. Mean ± SEM. Note the time scale (min) in the bottom of the figure which corresponds to the applied bed shear stress.

than 3 (P=0.01). There was a non-significant tendency for a higher number of suspended *Ascaris* eggs than *Trichuris,* however it was a non-significant borderline (P=0.053).

Figure 4 shows the results of the calculated erosion rates where an increase in bed shear stress resulted in a significant increase in erosion rate for particles (P<0.0001) and in a significant increase with a maximum in erosion rate for helminth eggs (P<0.0001). For particles the effect of day was highly significant (P<0.0001) showing that the erosion rate decreased over the three days. The interaction between shear stress and day was also significant (P<0.0001) which indicates that over the days it became more difficult to erode the particles and eggs. There was a significant difference between erosion rate of the two egg types, *Ascaris* and *Trichuris* (P=0.017).

The erosion threshold of the sediment on day 1, 2, and 3 was calculated on the basis of the plots shown in Figure 5 with values of 0.08 N m^{-2}, 0.06 N m^{-2}, and 0.09 N m^{-2}, respectively. The highest erosion rates on day 1 and 2 have been discarded from this plot (Fig. 5) since only the area of the first significant increase in erosion rate is important for determination of the erosion threshold. The erosion threshold for *Ascaris* eggs was 0.03 N m^{-2} for both day 2 and 3 whereas for *Trichuris* eggs it was 0.04 N m^{-2} on day 2 and 0.05 N m^{-2} on day 3.

3.3 Settling velocity of resuspended helminth eggs and particles

The settling velocity after applying a maximum bed shear stress of 0.5 N m^{-2} were calculated for the sediment, *Ascaris* eggs and *Trichuris* eggs for each of the days 1, 2, and 3 (Fig 6). There was a strong significant effect of time (day) on the settling velocity (P<0.0001). The settling velocity was significantly lower on day 2 compared to day 1 (P=0.004), on day 3 compared to day 2 (P=0.008), and on day 3 compared to day 1 (P<0.0001). The mean settling velocity of the suspended particles decreased over time from 1.3 ± 0.1 mm s^{-1} (±SD) on day 1 to 0.6 ± 0.1 mm s^{-1} on day 3. The mean settling velocity of *Ascaris* and *Trichuris* eggs also decreased over time, from 0.9 ± 0.3 mm s^{-1} and 1.0 ± 0.2 mm s^{-1} on day 1, respectively, to 0.5 ± 0.1 mm s^{-1} on day 3 for both egg types. There was no significant difference in settling velocity between the two helminth eggs, whereas the suspended sediment settled faster than both *Ascaris* eggs (P=0.016) and *Trichuris* eggs (P=0.047).

The results for settling velocity of helminth eggs in tap water on day 1 and day 3 are also shown in Figure 6 and ranged from 0.03 to 0.07 mm s^{-1}. There was no significant difference in settling

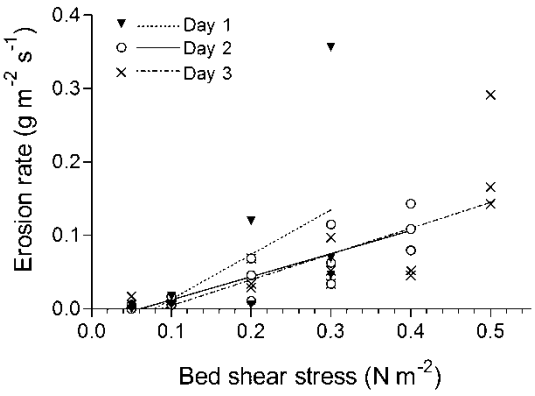

Figure 5. Plot used for calculation of the erosion threshold of the sediment on day 1, 2, and 3. The critical erosion rate chosen for determination of erosion thresholds was 0 g m^{-2} s^{-1}.

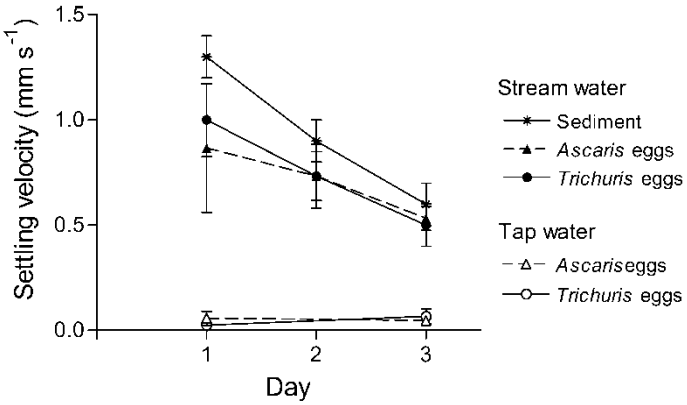

Figure 6. Median settling velocity of sediment and helminth eggs in stream water and helminth eggs in tap water.

velocity of the two helminth egg types and between day 1 and day 3. However, the interaction between water types (tap water or water with sediment) and day was highly significant ($P<0.0001$) showing much higher egg settling velocity in stream water with suspended particles than in tap water even though the settling velocity in stream water decrease from day 1 to 3.

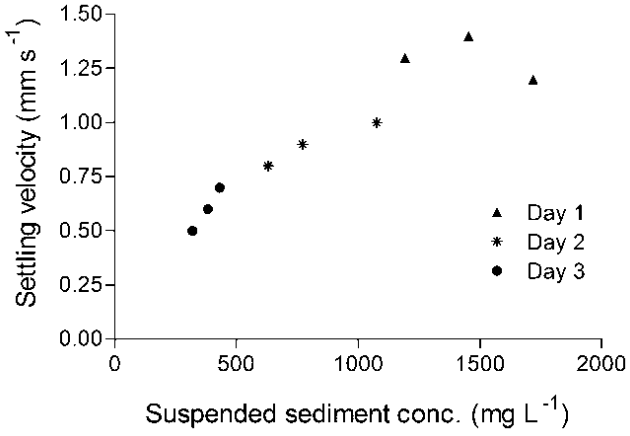

Figure 7. Median settling velocity of suspended sediment as a function of the initial SPMC of the same suspended sediment.

Figure 7 shows an overall positive relationship between the initial SPMC and the resulting settling velocity. From day 2 to 3 the initial SPMC decreased and hence also the settling velocity. The data points from day 1 do not support this tendency nonetheless there is a higher initial SPMC resulting in a higher settling velocity on day 1 as compared to day 2 and 3.

4. Discussion

In this study, erodibility of both sediment and helminth eggs decreased over time which indicates that consolidation of the sediment takes place along with adhesion of eggs to sediment particles incorporating the eggs in the sediment. This is further supported by the settling velocity

experiments where a much higher settling velocity was found for helminth eggs associated with particles as compared to eggs in clean tap water. The increased erosion resistance with time may also have been partly caused by biological interactions with the sediment, but none of the analyzed physical and biological properties of the sediment beds showed significant changes over time. However, this is most likely caused by the relatively short experimental period of only three days as other laboratory studies with longer time span (> 1 week) have shown marked biological impact on erodibility of cohesive sediment (Andersen *et al.*, 2005; Lundkvist *et al.*, 2007). Typical biological effects are formation of biofilms by benthic diatoms (Lundkvist *et al.*, 2007) and bioturbation of sediment by macro-fauna (Andersen *et al.*, 2005). The incorporation of eggs into sediment aggregates indicates that such biological effects will also influence the erosion and settling characteristics of helminth eggs. The impact on erodibility of helminth eggs is that much higher bed shear stresses is needed for erosion of eggs incorporated into the sediment matrix compared to individual eggs resting on the bed.

The settling experiments showed that when the helminth eggs settle without sediment the settling velocity is about an order of magnitude lower as compared to a situation with eggs settling in a suspension of fine-grained cohesive sediment (Fig. 6, day 1). This shows that the eggs interact with the suspended sediment and gets incorporated into sediment aggregates, and hence increase their settling velocity. The aggregation of suspended sediment is clearly visible through the fact that the settling velocity is twice as high as would be calculated on the basis of the primary grain size distribution using Stoke´s Law (0.73 mm s^{-1}). The observed effect of suspended material on settling of helminth eggs confirms the observations from a previous study (Sengupta *et al.*, 2011) in that the eggs interact with other particles in suspension. Sengupta *et al.* (2011) observed both slightly lower settling velocities when eggs settled in a suspension of raw wastewater compared to settling in clean water, whereas in this study the settling velocity was increased due to aggregation with cohesive sediment. The present study which made use of fine-grained sediment collected in a small natural stream is likely a closer approximation to the situation in the majority of water bodies, i.e. irrigation channels, streams etc. In such channels and streams natural fine-grained sediment will dominate, as opposed to sewage particles, except for sites very close to sewage outlets, due to presence of fine-grained material in channel banks and surrounding fields. The present study also differs from the study of Sengupta *et al.* (2011) in that the effect of sedimentation and aggregation at the bed was studied. The larger increase in settling velocity of the eggs found in our study demonstrates the

importance of sediment aggregation at the bed, an effect which is far less studied than flocculation in the water column (Andersen and Pejrup, 2002). Our results document that the typical effect of suspended material on egg settling will most likely lead to an increase in the settling velocity due to particle interaction and aggregation both in the water column and at the bed.

The settling velocity of the suspended sediment is partly determined by the initial concentration of suspended sediment (Fig 7), indicating that sediment aggregation also takes place in the water column (flocculation), even at the short time-scale of these experiments. This correlation between SPMC and settling velocity is very common in nature (e.g. van Leussen, 1996) and since the erosion rate decreases with time (less SPMC with time), the settling velocity also decrease over time in the experiments. Visual inspection of the suspended aggregates at low bed shear stresses (< 0.3 N m^{-2}) showed an increase in aggregate size with time, but the decreasing settling velocity over time after 0.5 N m^{-2} indicates that the larger resuspended aggregates do not remain intact in the turbulence generated by the propeller at the higher bed shear stresses. Consequently, the decreasing settling velocity of the suspended sediment and associated eggs over time is an effect of both the decreasing erodibility of the sediment with time and the experimental set-up with settling from a rather high level of applied bed shear stress destroying the resuspended aggregates. In nature the temporal variation is expected to be opposite with increasing settling velocity over time due to increasing sediment aggregation (Andersen and Pejrup, 2002).

The adherence to and incorporation into the sediment bed decrease the mobility of the eggs and the aggregation increases the effective settling velocity of helminth eggs which also results in reduced mobility. This study shows that helminth eggs should not be viewed as single entities in water systems when modeling and predicting the distribution and fate of eggs since both erodibility and settling velocity of helminth eggs primarily are determined by the mobility of the sediment present in the water canal or stream. If this interaction is not taken into account, mobility and spreading of the eggs may be overestimated. It could also be speculated that the mobility of helminth eggs may be even more decreased due the sticky surface of especially *Ascaris* eggs (Jimenez, 2007). It has been proposed that the uterine-derived outer-layer is responsible for the sticky appearance of *Ascaris* eggs (Fairbairn, 1957). However, there is no evidence for such an egg surface property and the reason for the wide acceptance of eggs being sticky may be that for experimental purposes eggs derived from the female worm uteri are often used. When eggs pass through the intestine of the host

the colorless uterine layer becomes stabilized by a quinine-tanning. Eggs from the worm uterus have not yet become tanned and clump together if not treated with sodium hypochlorite, NaClO, or sodium hydroxide, NaOH (Nejsum *et al.*, 2009). *Ascaris* eggs obtained from feces are tanned and less sticky (Roepstorff, 2003), and would also be better representatives of eggs found in the environment with regard to surface properties. Besides the attributed sticky appearance of eggs, physical-chemical surface properties probably determine adhesion of eggs to surfaces and particles. The mechanism and determinants of adherence of helminth eggs are not well understood and may, like for other particles, depend more on the physical-chemical surface properties which are determined by pH, temperature and the ionic composition of the surrounding water or soil.

When constructing water channels, e.g. for irrigation purposes, it is important to control the water flow to deliver the correct amount of water to the irrigated fields as well as to limit erosion of the canal sediment and banks hindering downstream blocking by deposited sediment and collapse of banks (Brouwer *et al.*, 1985). Resuspension of both *Ascaris* and *Trichuris* eggs occurred in our study even at the lowest bed shear stress applied supported by the erosion thresholds which was determined to be $\leq$0.05 N m^{-2} for newly deposited eggs. The maximum erosion threshold of approximately 0.1 N m^{-2} for the sediment and 0.05 N m^{-2} for the eggs can be recalculated to a mean flow velocity in open irrigation channels if the Manning number M (roughness coefficient) is known. Using a Manning M of between 33 and 55 for channels in bare soils without vegetation (Engelund and Pedersen, 1978) and a water depth of 1 m, the critical depth-average mean flow velocity is between 0.10 and 0.18 m s-1 for the sediment and 0.07 and 0.12 m s-1 for the eggs. These velocities are well below typical design flow speeds for irrigation channels, and helminth eggs will consequently show high mobility if introduced into typical irrigation channels. Based on our results, removal of eggs from irrigation water by sedimentation will therefore only take place in rather stagnant water, i.e. ponds and lakes. Entrapment of eggs by vegetation in the water stream or channel will occur and egg numbers will also decrease when eggs are eaten by fish or macro-fauna on the sediment bed. However, the extent of such reductions is uncertain. On the other hand, resuspension of deposited helminth eggs will also occur as a result of increased water velocity during heavy rain events and pumping of water. These and other environmental factors must be taken into account when developing models for sedimentation of helminth eggs in irrigation canals.

5. Conclusion

The study has shown that helminth eggs interact both with sediment at the bed and suspended sediment. The eggs adhere to the fine-grained particles on the bottom and are eroded along with the sediment and subsequent flocculation in the water column further increases their aggregation. This adherence to and incorporation into the bed decrease the mobility of the eggs and the aggregation increases the effective settling velocity of helminth eggs which also results in reduced mobility. Our findings show that helminth eggs should not be viewed as single entities in water systems when modeling and predicting the distribution and fate of eggs since both erodibility and settling velocity of helminth eggs primarily are determined by the mobility of the sediment present in the water canal or stream. If this interaction is not taken into account, mobility and spreading of the eggs will most likely be overestimated. In this study, recalculation of the erosion threshold for helminth eggs and sediment to a mean flow velocity in open irrigation channels showed that even at relatively low current velocities the eggs will demonstrate high mobility if introduced into typical irrigation channels.

Acknowledgement

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4.2.1 Additional results and discussion from the resuspension experiments (**Paper II**)

Prior to calculation of settling velocities (as described in section 3.3.2), a log-plot of suspended particulate matter concentration (SPMC) or egg numbers as a function of settling time was done for graphical inspection of the exponential dependency (showing as a straight line in the log-plot). Mean values of SPMC and egg numbers for three experimental cores on day 2 and 3 were plotted (see Figures 4.1 and 4.2). Figure 4.1 shows the straight line for suspended sediment during the six minutes of settling. However, only data points for time 0 min and 6 min were plotted due to errors in calibration of the intermediate time points measured with the optical back-scattering sensor. From Figure 4.2 it is seen that for *Ascaris* and *Trichuris* eggs the line is not entirely straight since the slope of the graph decreases after 3 min. Thus the settling velocity is higher in the first 3 minutes than in the last 3 minutes. This indicates that not all eggs settle with the same velocity, most likely because of a certain distribution of the egg size (larger eggs settling faster than smaller eggs). However, the settling velocity of both suspended sediment and helminth eggs was calculated based on 6 minutes of settling to be able to compare the two.

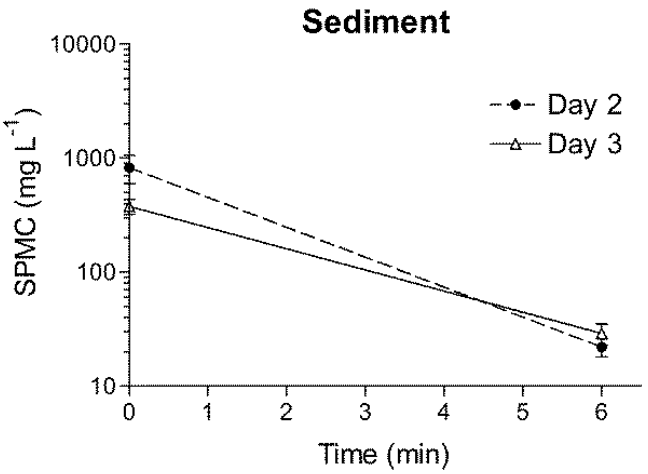

Fig. 4.1 Plot of suspended particulate matter concentration (SPMC) as a function of settling time on day 2 and 3. Note the log-scale on Y-axis.

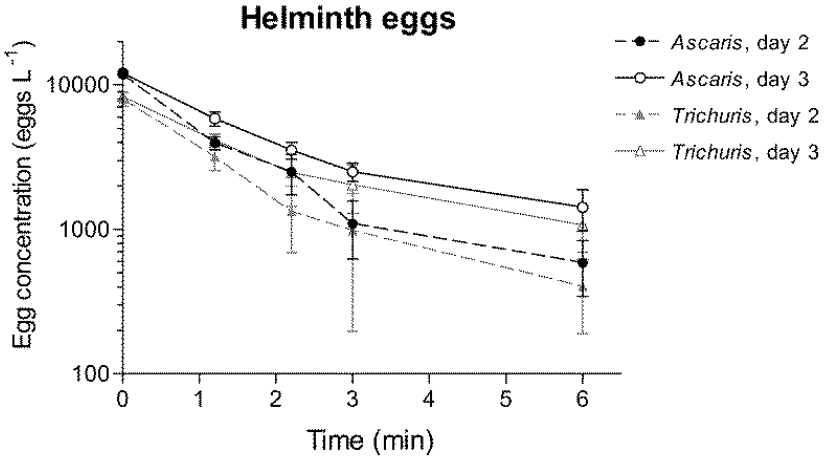

Fig 4.2 Plot of the concentration of suspended eggs (*Ascaris* and *Trichuris*) as a function of settling time on day 2 and 3. Note the log-scale on the y-axis.

4.3 Paper III

Use of *Moringa oleifera* seed extracts to reduce helminth eggs and turbidity in irrigation water

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Use of *Moringa oleifera* seed extracts to reduce helminth eggs and turbidity in irrigation water

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Abstract

Water from wastewater-polluted streams and dug-outs is the most commonly used water source for irrigation in urban farming in Ghana, but helminth parasite eggs in the water represent health risks when used for crop production. Conventional water treatment is expensive, requires advanced technology and often breaks down in less developed countries so low cost interventions are needed. Field and laboratory based trials were carried out in order to investigate the effect of the natural coagulant *Moringa oleifera* (MO) seed extracts in reducing helminh eggs and turbidity in irrigation water, turbid water, wastewater and tap water. In medium to high turbid water MO extracts were effective in reducing the number of helminth eggs by 94-99.5% to 1-2 eggs per litre and the turbidity to 7-11 NTU which is an 85-96% reduction. MO is readily available in many tropical countries and can be used by farmers to treat high turbid water for irrigation, however, additional improvements of water quality, e.g. by sand filtration, is suggested to meet the guideline value of ≤ 1 helminth egg per litre and a turbidity of ≤ 5 NTU as recommended by World Health Organization. A positive correlation was established between reduction in turbidity and helminth

eggs in irrigation water, turbid water and wastewater treated with MO. This indicates that helminth eggs attach to suspended particles and/or flocs facilitated by MO in the water, and that turbidity and helminth eggs are reduced with the settling flocs. However, more experiments with water samples containing naturally occurring helminth eggs are needed to establish whether turbidity can be used as a proxy for helminth eggs.

Keywords: Low quality water treatment, *Moringa oleifera*, helminth eggs, turbidity, urban vegetable farming.

1. Introduction

It is estimated that about 20 million hectares of agricultural land worldwide, including urban farming areas, are irrigated with untreated, partially treated or untreated wastewater (Jimenez and Asano, 2008; Raschid-Sally and Jayakody, 2008). In Ghana, the most commonly used sources of irrigation water in urban farming are water from wastewater-polluted streams, dug-outs and stormwater. Farmers use these water sources since they are free and reliable allowing for all-year-round farming (Obuobie *et al.*, 2006). Market-oriented and popular vegetables such as lettuce, cabbage, green pepper and spring onions are often grown. This practice is important for the livelihood of many urban dwellers and a means of attaining balanced diets and urban food security. In Accra, Ghana, it is estimated that there are 800-1000 urban vegetable farmers (Keraita and Drechsel, 2004), and the vegetables produced are eaten by about 200,000 urban residents daily (Amoah *et al.*, 2007). On a global scale, at least one tenth of the world's population is thought to consume foods produced by irrigation with wastewater (Smit and Nasr, 1992).

Irrigation water sources in less developed countries are often contaminated with untreated wastewater since large volumes of wastewater generated in urban areas are not treated (Amoah *et al.*, 2005). When untreated wastewater is used for irrigating crops, it poses substantial risks to human health, not only for farmers, but also for surrounding communities and consumers of the crops, in particular when crops are eaten uncooked (Blumenthal *et al.*, 2000). The major health risks from irrigation with untreated, partially treated and occasionally treated wastewater are associated with the viral, bacterial and parasite pathogens (Feachem *et al.*, 1983; Shuval *et al.*, 1986). Risk assessments done in urban vegetable farming sites in Ghana show fecal contamination levels in irrigation water with 5-10 helminth eggs per litre of water (Obiri-Danso *et al.*, 2005; Amoah *et al.*,

2011). Helminth parasite eggs in wastewater are of particular health concern due to the high burden of helminth diseases in less developed countries where use of untreated wastewater is most prominent. The most important helminth eggs include *Ascaris lumbricoides* (human roundworm), *Trichuris trichiura* (human whipworm), and *Ancylostoma duodenale*/*Necator americanus* (two human hookworms) (Feachem *et al.*, 1983; Ensink *et al.*, 2008). It is estimated that at least 1.2 billion people globally are infected with one or more of the mentioned species of intestinal helminths (Bethony *et al.*, 2006; de Silva *et al.*, 2003).

Helminth eggs are extremely resistant to environmental stress (Wharton, 1980) and a high degree of egg removal is therefore required by wastewater treatment if wastewater is to be used safely in irrigated agriculture (Stott, 2003). Recent guidelines by the World Health Organization (WHO) recommend that wastewater used for irrigation should contain ≤ 1 helminth egg per litre when no other risk reduction options are available (WHO, 2006). High concentration of suspended particles in water impede irrigation by drippers and sprinklers and also affect the quality of crops produced (Pescod, 1992). Thus, in a recent study in Ghana turbidity levels as high as 791 NTU were recorded (Keraita *et al.*, 2008). Therefore WHO recommends that irrigation water has a turbidity of 5 NTU (WHO, 2006). An intervention suitable for removing helminth eggs as well as reducing turbidity in low quality irrigation water would benefit many farmers.

Research on low-cost interventions has been conducted in Ghana and elsewhere to identify sustainable interventions for reducing risks in urban vegetable farming using low quality water for irrigation as conventional wastewater treatment, which could be more effective, is expensive to install and maintain (Keraita, 2008; Carr and Strauss, 2001). The tested methods included farm–based measures to improve water quality using sedimentation ponds and slow sand filters as well as reducing pathogen levels on crops through improved irrigation methods and enhanced pathogen die-off on crops by withholding irrigation before harvest. These low-cost intervention measures have shown great potential but there are still need for local adapted low-cost technologies to treat water for irrigation.

Studies have shown that treatment of water with *Moringa oleifera* (MO) extracts can achieve 1-4 log unit reduction of pathogens, i.e. fecal bacteria and *Schistosoma mansoni* cercariae (Olsen, 1987; Madsen *et al.*, 1987). In Ghana, a study conducted on surface water used for domestic purposes

showed a 90-99% reduction in fecal coliform levels (Boateng, 2001). However, no data exist on the effect of MO extract on removal of helminth eggs from irrigation water. In addition, though most studies have focused on treatment of drinking water, which has low turbidity, the MO extracts appear effective in treating high-turbidity water with turbidity reduction of more than 95% (Muyibi and Alfugara, 2003). The aim of the present study was to investigate under field and laboratory conditions the ability of MO seed extracts to reduce the number of helminth eggs and turbidity in different types of water.

2. Materials & methods

Experiments for removal of helminth eggs and reducing turbidity were carried out under typical field conditions in Ghana where urban farmers use wastewater-polluted water for irrigation and under controlled laboratory conditions in Denmark. Experiences obtained from the field-based studies in Ghana with extraction of coagulant from *Moringa oleifera* (MO) seeds and the effects of adding different concentrations of coagulant on water quality supported the design of the laboratory experiments. In Ghana, helminth eggs were present in irrigation water which was contaminated with untreated domestic wastewater. In the laboratory, a known quantity of helminth eggs was added to the different water types used for the experiments.

2.1 Study site and water types

The field experiments were done at Karikari, a prominent urban vegetable farming site in Kumasi, Ghana. Water samples (irrigation water) were taken from on-farm ponds (dug-outs) which received wastewater from nearby households and were used for irrigation at the time of the study.

Laboratory experiments were conducted to model field conditions where water used for irrigation may show high variation in pollution levels, e.g. in water turbidity. Three water types with low turbidity (< 50 NTU; tap water), medium turbidity (50-150 NTU; wastewater) and high turbidity (>150 NTU; turbid water) were used (Lea, 2010). The tap water was drinking water used by residents of Copenhagen supplied by Frederiksberg Municipality Water Supply, Denmark. The wastewater was obtained from the inlet canal to the primary settling tanks at the wastewater treatment plant “Lynetten”, Copenhagen, Denmark. Turbid water was collected from a natural freshwater stream (GPS: 55.97428, 12.42760) in Northern Sealand, Denmark and left to settle to turbidity levels of about 200 NTU before being used in the experiments.

Initial examinations of tap water, wastewater and turbid water by a modified McMaster method (Sengupta *et al.*, 2011) showed no presence of helminth eggs. Eggs of *Ascaris suum* and *Trichuris suis* were recovered from feces of naturally infected Danish pigs and used in the laboratory experiments. The pig helminths, *A. suum* and *T. suis* are often used as model organisms for the corresponding human helminth parasites, *A. lumbricoides* and *T. trichiura* (Boes and Helwigh, 2000). The eggs of pig helminths are virtually identical in morphology and size to the corresponding human parasite eggs and are relative easy to obtain in high numbers from infected pigs. Eggs were isolated by sieving fresh feces through a series of sieves followed by flotation of eggs (Sengupta *et al.*, 2011). A total of 2100-2200 eggs of each helminth type were added to individual jars (one litre of each water type) with a pipette under slow stirring (see introduction to the jar test in section 2.2).

2.2 Extraction of coagulant from MO seeds and determination of optimum dosage

MO seeds used for coagulation in both field and laboratory experiments were obtained from a commercial seed supplier in Kumasi, Ghana. Mature seeds showing no signs of discoloration, softening or extreme desiccation were used. The shell enveloping the seeds in the pods was removed and the non-shelled seeds were stored for maximum six months in dry conditions until use. Husks covering the MO seeds were manually removed before extraction and the kernel grounded using a kitchen mortar to a fine powder. The powder was mixed with tap water (Ndabigengesere and Narasiah, 1998) to reach a final concentration of 3% or 5% weight per volume (w/v) suspension. The mixture was stirred at 300 rpm for 30 min using a magnetic stirrer, to promote extraction of the coagulant proteins, and filtered through sieve cloth with a mesh size of 15 µm. The filtrate was used as coagulant. In order to prevent any aging effects due to storage (Katayon *et al.*, 2006), a fresh MO solution was prepared on each experimental day and the solution was shaken vigorously before use. Coagulation efficiency of MO extract was assessed using the jar test (Muyibi and Alfugara, 2003; Ndabigengesere *et al.*, 1995). Each of six glass jars was filled with one litre of water from the same source, stirred by a propeller and added the coagulant (MO+). The optimum MO extract dosage was determined by adding different volumes of the 3% or 5% MO extract (20, 40, 60, 80, 100, and 120 ml) to the irrigation water in the field study. After initial trials lower volumes (4, 8, 16, and 32 ml of 5% w/v MO extract) were tested in the laboratory experiments. After adding the coagulant, the propeller was stopped completely and the content of the water left to coagulate and sedimentate. A set of jars without addition of coagulant (MO-) were

used as controls. The optimum dosage of MO extract was the volume and concentration where no further reduction in turbidity was observed when more MO extract was added.

2.3 Experimental design and sampling

In the field experiments in Ghana, samples were taken in triplicates, three times per week for five consecutive weeks yielding a total of 45 water samples. Each water sample was divided into two sub-samples with one sub-sample used as control (MO-) and the other sub-sample divided into six one litre samples poured into the jars. The water sample was then mixed with the optimum dosage of MO extract. After the coagulation process, samples (MO+) of the supernatant were collected in triplicates at different time intervals i.e. after 30 min, 1 hr, 1.5 hrs, 2 hrs, 2.5 hrs and 3 hrs, with each jar representing a time point. These samples were analyzed for helminth eggs and turbidity following standard procedures as detailed in section 2.4.

In the laboratory experiments with the low, medium and high turbidity water types (section 2.1) six water samples were collected of each water type. Each water sample was divided into two with one sub-sample further divided into three one litre samples which were used as controls (MO-). The other sub-sample was divided into six one litre samples poured into the jars. The water sample was then mixed with the optimum dosage of MO extract. After the coagulation process, samples (MO+) of the supernatant were taken at different time intervals, i.e. after 15 min, 30 min, 45 min, 1 hr, 1.5 hrs, and 2 hrs, with each jar representing a time point. The samples were analyzed for helminth eggs and turbidity as described in section 2.4. In the laboratory experiments helminth eggs were enumerated in the sediment collected from the jars to determine the total recovery of added *Ascaris* and *Trichuris* eggs.

2.4 Helminth eggs and turbidity

Helminth eggs in the irrigation water used in field experiments in Ghana were enumerated using the USEPA modified concentration method (Schwartzbrod, 1998) and identified to species using the WHO Bench Aid for identifying helminth eggs (WHO, 1994). In the laboratory experiments in Denmark, a modification of the concentrated McMaster method (Sengupta *et al.*, 2011) was used for counting *Ascaris* and *Trichuris* eggs.

Samples for turbidity measurements were collected from the supernatant in different jars using a standard pipette. In field experiments, the turbidity was recorded with a Cyberscan IR TB100 turbidimeter (EUTECH Instruments, Singapore). For the laboratory experiments in Denmark, the turbidity was recorded with a TN-100 turbidimeter (EUTECH Instruments, Singapore).

2.5 Statistical methods

Data from the field experiments in Ghana were analyzed by two-way ANOVA of the log-transformed concentrations of helminth eggs and non-transformed NTU turbidity data with addition of MO and time as fixed effects. Data from the laboratory experiments in Denmark were analyzed by two-way ANOVA of the log-transformed concentrations of *Ascaris* eggs, *Trichuris* eggs and turbidity with addition of MO and time as fixed effects for each of the three water types separately. The responses at time 0 was excluded from the statistical analyses in the laboratory experiments since they are not measured data points. For both data sets, the non-significant effects were removed by backward model reduction on a 5% significance level, and post-hoc tests with Tukey-HSD correction for multiple testing were done when interactions was significant and to verify the significance of differences among the means. The model was validated by residual- and normal quantile-plots. Linear regression was performed on helminth egg number as a function of turbidity for irrigation water, turbid water and wastewater.

Table 1. Characteristics of the water types used in field experiments in Ghana and in laboratory experiments in Denmark.

Parameter	Field experiment	Laboratory experiment		
	Irrigation water	Turbid water[a]	Wastewater[a]	Tap water[b]
pH	5.5-7.3	6.8-7.5	7.1-7.3	7.3-7.5
Turbidity (NTU)	42-183	199-221	73.6-77	0-0.8
Conductivity (mS/m)	10.5-50	N.D.	N.D.	78.6-137
Dissolved solids (mg/L)	110-500	427	N.D.	N.D.
Suspended solids (mg/L)	N.D.	153	289	N.D.
TOC (mg/L)	N.D.	17.5	N.D.	N.D.
COD (mg/L)	N.D.	10.6	540	N.D.
BOD (mg/L)	N.D.	1.4	169	N.D.
Alkalinity, $CaCO_3$ (mg/L)	60-150	476.4	N.D.	N.D.
Total N (mg/L)	1.4-5.4	7.86	47.9	N.D.
Total P (mg/L)	1.3-11.9	0.73	8.03	<0.015
Chloride, Cl^- (mg/L)	6-20	62.8	N.D.	113-135
Fecal coliforms (/100 ml)	10^6-10^7	N.D.	N.D.	<1

[a]Water characteristics according to the wastewater treatment plant "Lynetten", Copenhagen, Denmark.
[b]Water characteristics according to the Frederiksberg Municipality Water Supply, Copenhagen, Denmark.
Not determined = N.D.

3. Results

Characteristics of the irrigation water used in the field experiments in Ghana and the three types of water used in the laboratory experiments in Denmark are shown in Table 1.

3.1 Optimum dosage of MO extract

Figure 1 shows the effect of different volumes of 3% and 5% MO extract on turbidity in irrigation water. The optimum dosage of MO extract was highly dependent on the types of water and their characteristics. The optimum dosage using 3% w/v was 100 ml/litre for the irrigation water whereas much lower levels of 5% w/v were found for turbid water (8 ml/litre) and for wastewater (4 ml/litre) (data not shown). For tap water, 5% w/v MO extract was selected with 8 ml/litre for comparison analysis.

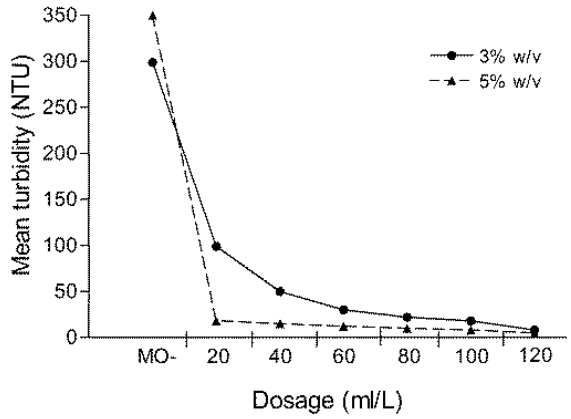

Fig. 1. The effects of different volumes of MO extract (3% and 5%) on reducing turbidity in irrigation water used for field irrigation in Ghana. On the X-axis MO- indicates that water is not treated with MO.

3.2 Effect on reduction of turbidity

Field experiments showed a clear effect of MO on turbidity with time (Figure 2A). The turbidity continued to decrease until sampling time 90 min where 92% of the initial turbidity was reduced and gradually remained constant until 180 min. Therefore the optimum settling time obtained was 1.5 hrs (9 NTU).

The results from laboratory experiments are shown in Figures 2B-2D. The effect of adding MO extract significantly lowered the turbidity ($P<0.0001$) in turbid water and wastewater (Fig. 2B and 2C), whereas in tap water the turbidity increased with MO treatment (Fig. 2D). The effect of time was highly significant for both turbid water ($P=0.0006$) and wastewater ($P=0.0078$) and the interaction between MO and time was also significant ($P=0.0036$-0.0473). The latter demonstrates that turbidity generally decreases with time, but faster when MO was added. For both turbid and wastewater, the reduction in turbidity from 60 min to 120 min was almost negligible both with and without MO treatment, as also seen in the field experiments. In that one hr period, 96% of the initial turbidity in turbid water was reduced by adding MO compared to 82% removal in water without MO. The corresponding reduction in wastewater was 85% and 54%, respectively. When treating

with MO, the optimum reduction in NTU occurred in turbid water after a settling time of 120 min (6.5 NTU), while in wastewater this reduction was seen after 90 min of settling (10.8 NTU).

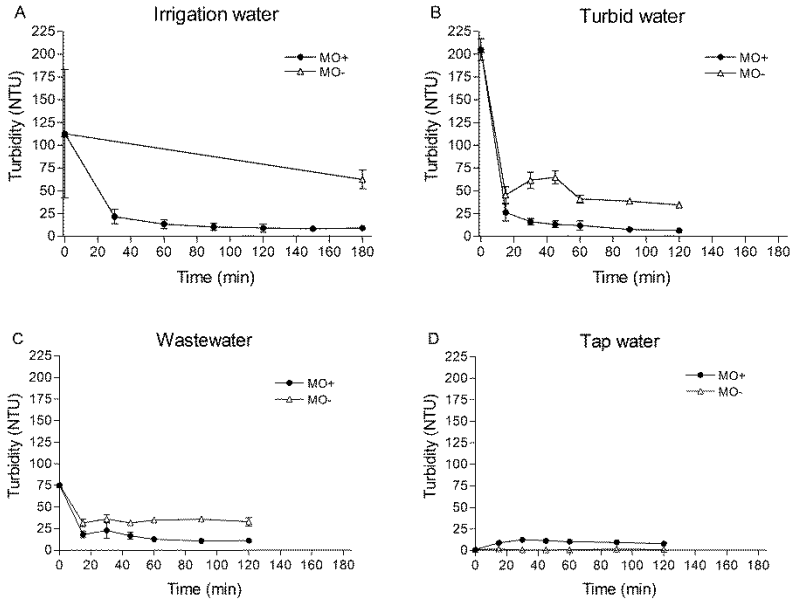

Fig. 2. The effect of *Moringa oleifera* (MO) extract treatment on turbidity in irrigation water (A), turbid water (B), wastewater (C), and tap water (D). Error bars are standard deviation.

3.3 Effect on removal of helminth eggs

Results of helminth egg removal by MO extracts are shown in Figure 3. In field studies, natural sedimentation (MO-) for three hrs removed helminth egg numbers by 24%, from 14.0 to 10.6 eggs per litre. When sedimentation was enhanced with MO, the reduction was by 94%, from 14.0 to 0.8 eggs per litre which was significantly higher ($P<0.05$) than the reduction without MO (Fig. 3A). When treated with MO, the number of helminth eggs in irrigation water reduced exponentially, $y = 22.8e^{-0.5x}$ (where y = no. of helminth eggs, x = time in hrs), to less than 1 egg per litre. Optimum removal was achieved after 2-2.5 hrs (1.0-1.4 eggs per litre) of sedimentation, after which no significant reduction of helminth eggs was recorded. Eggs of *Ascaris* spp. (>75%) were the most common helminth species found in the irrigation water, followed by *Trichuris* spp., *Fasciola* spp. and *Schistosoma* spp. eggs.

For laboratory experiments (Fig. 3B-3G), the overall removal of both *Ascaris* and *Trichuris* eggs from both turbid, wastewater and tap water were significantly higher when treated with MO ($P<0.0001$-0.006) compared to not treating with MO. The effect of time was also highly significant ($P<0.0001$) for both egg types. In turbid water, a total of 96.7% of *Ascaris* eggs was removed after 15 min with or without MO treatment. From 60 to 120 min, the reduction was more or less the same with a reduction from 99.5 to 99.9% (0.7 eggs per litre). The same tendency was seen for *Trichuris* eggs in turbid water. Here 93.9% was removed after 15 min with MO treatment as compared to

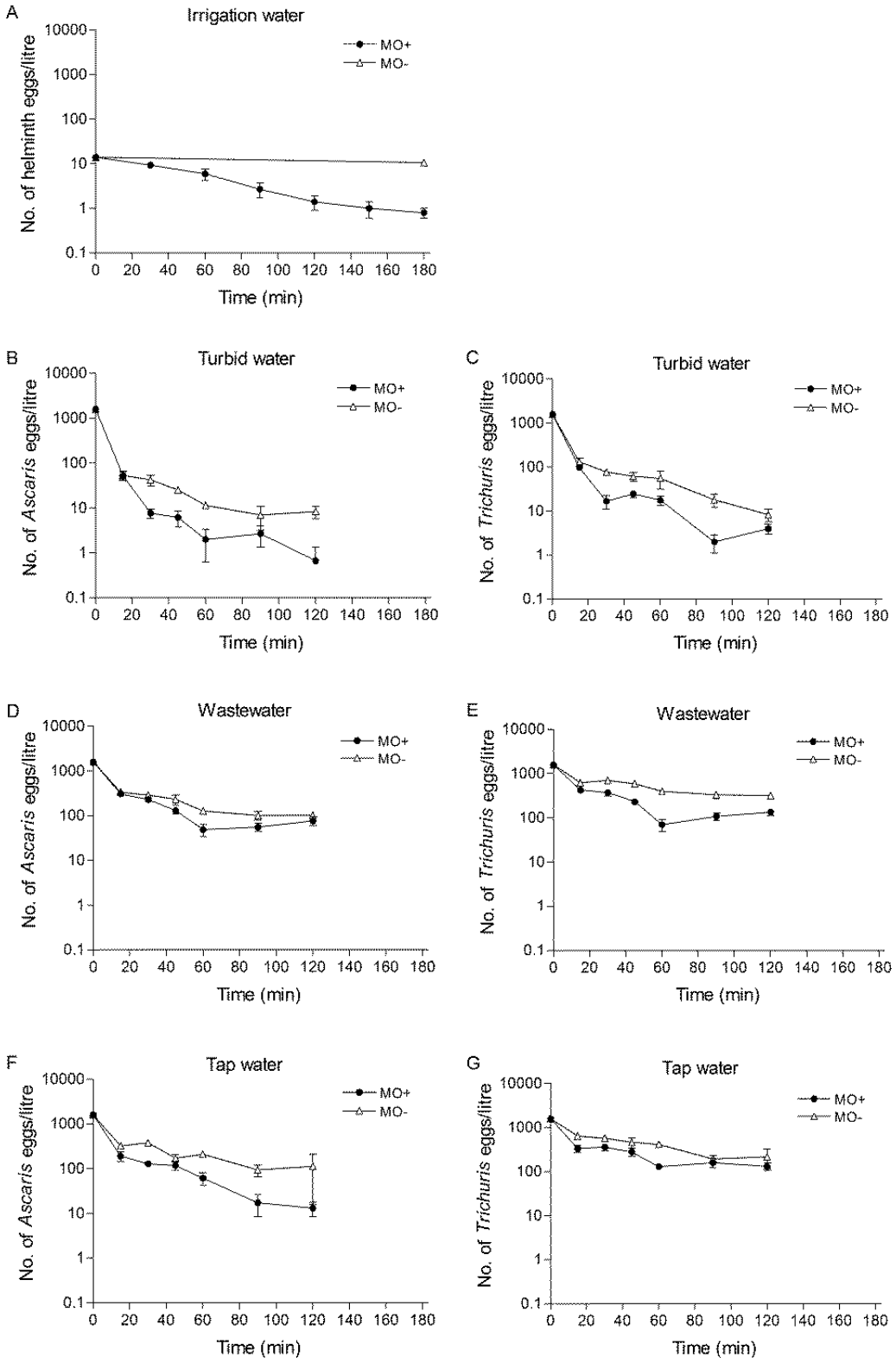

Fig. 3. The effect of *Moringa oleifera* (MO) extract treatment on removal of helminth egg numbers in irrigation water (A), turbid water (B-*Ascaris*, C-*Trichuris*), wastewater (D-*Ascaris*, E-*Trichuris*), and tap water (F-*Ascaris*, G-*Trichuris*). Error bars are standard error. Note the log-scale on Y-axis.

91.9% without MO treatment. After 60 min, the corresponding figures were 99.5% (2 eggs per litre) with MO and 98.3% without addition of MO extract. Optimum reduction of *Ascaris* eggs in turbid water treated with MO was obtained after 60 min (2.0 eggs per litre) and after 90 min (2.0 eggs per litre) for *Trichuris* eggs. In wastewater, the removal of *Ascaris* eggs after 15 min was 80.7% with MO and 79.0% without treatment. The removal of *Trichuris* eggs from wastewater was 73.3% after 15 min with MO treatment, while 60.9% was removed in water without MO extract. After one hr, the optimum egg reduction was obtained for both egg types (48.8 *Ascaris* eggs per litre; 87.2 *Trichuris* eggs litre). In tap water, 96.1% *Ascaris* and 91.8% *Trichuris* eggs were removed after 60 min of treatment with MO, while 86.9% and 74.0%, respectively, were removed in tap water without MO. The optimum removal of *Ascaris* eggs in tap water (17.3 eggs per litre) was seen after 1.5 hrs and for *Trichuris* eggs (130.5 eggs per litre) it was achieved after one hr. For *Ascaris* eggs, the reduction in tap water which had lower turbidity was surprisingly higher than in wastewater.

In the laboratory experiments, total recovery of *Ascaris* and *Trichuris* eggs was higher in samples treated with MO as compared to samples without MO extract. The average recovery was 93% and 74% for *Ascaris* eggs, and 92% and 68% for *Trichuris* eggs with and without addition of MO extract, respectively.

3.4 Relationship between turbidity and helminth eggs

A positive correlation was found between turbidity and number of helminth eggs in irrigation water, turbid water and wastewater treated with MO extract (Figure 4). When all three water types were treated with MO the slope of the regression line was significantly different from zero ($P<0.0001$) meaning that the linear relationship between turbidity and helminth eggs is highly significant. However, the strength of the correlation varied for the different water types. The weakest correlation was seen in the field study with irrigation water where 9% ($r^2=0.09$) of the variability in helminth egg numbers was associated with the variability in turbidity, whereas the strongest correlation was found for turbid water where 53-62% ($r^2=0.53$-0.62) of the variability could be ascribed to the relationship with turbidity. In water types used as controls (MO-), no correlation was found indicating that addition of MO affects the relationship between turbidity and helminth eggs.

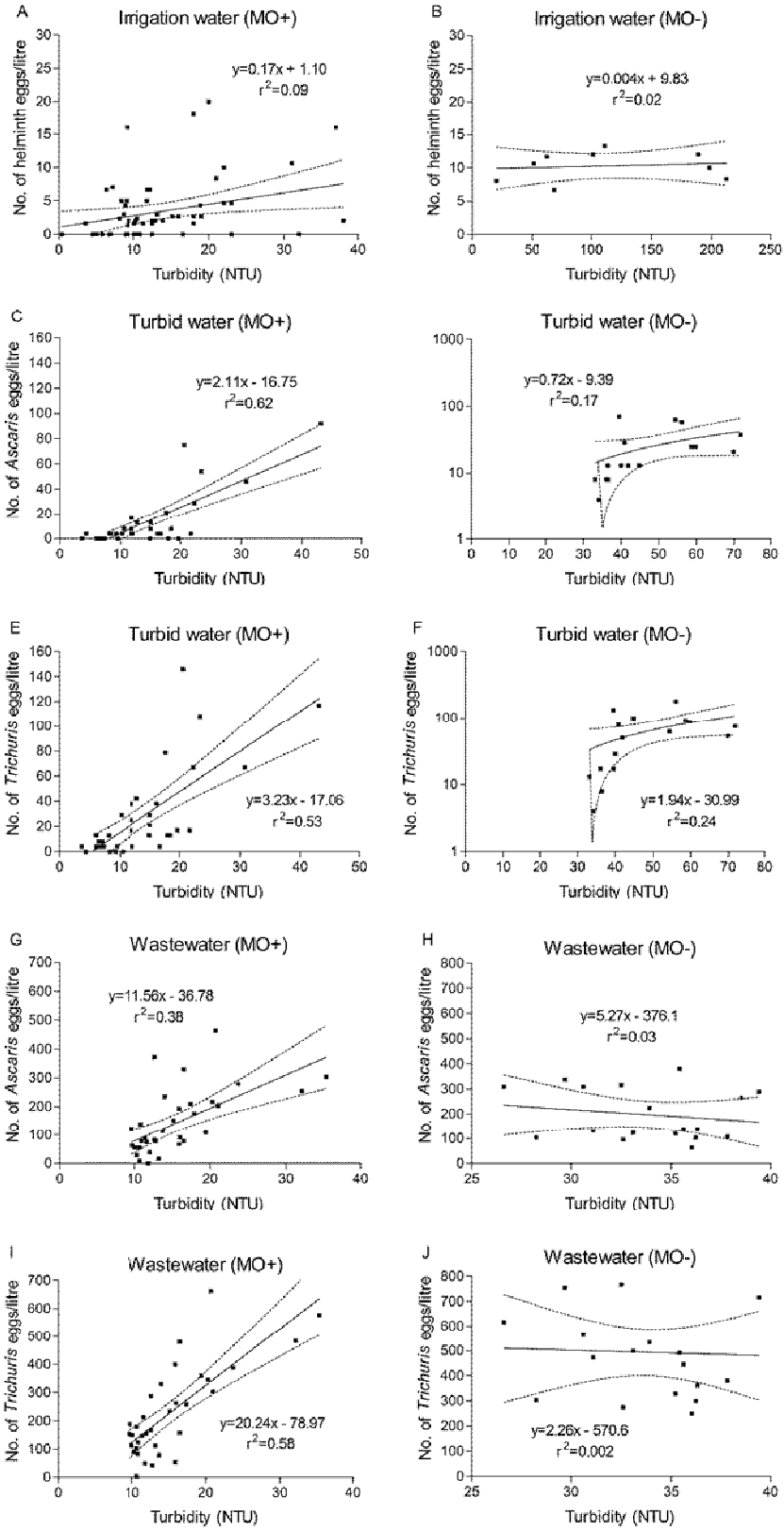

Fig. 4. Correlation between helminth eggs and turbidity following *Moringa oleifera* (MO) treatment in irrigation water (A-B), turbid water (*Ascaris*: C-D; *Trichuris*: E-F), and wastewater (*Ascaris*: G-H; *Trichuris*: I-J). Straight line is regression line with 95% confidence zones (dotted lines).

4. Discussion

4.1 Reduction of turbidity

In this study, it was observed that MO extracts reduced turbidity in high turbid water (>150 NTU) to 6.5 NTU which is a 96% reduction. In medium turbid water (50-150 NTU) the reduction in turbidity was 85-92% to 9-10.8 NTU. Muyibi & Evison (1995) also tested MO extract to reduce turbidity and found a reduction of up to 98.5% in water samples with initial turbidity of 600 NTU. Other studies have found similar (80-99%) reductions following MO treatment of different water types (Muyibi and Alfugara, 2003; Jahn, 1988). Removal of turbidity is achieved by water soluble cationic proteins in the MO seed extracts which enhance coagulation and flocculation (Muyibi and Evison, 1995; Jahn, 1988; Folkard and Sutherland, 2001). One mechanism of action is that positive charged MO proteins bind to the negative charged suspended particles by coulomb forces neutralizing the surface charge, i.e. colloidal charge neutralization, and reduced electrostatic repulsion leads to flocculation of particles, i.e. bridging of destabilized particles. Another mechanism of action is that positive charged MO proteins bind to parts of the surface of the negatively charged particles (by adsorption), and due to particle collision and interparticulate saturation of the differently charged particle surface areas flocs are formed (Ndabigengesere *et al.*, 1995; Bhatia *et al.*, 2007a; Bhatia *et al.*, 2007b; Gassenschmidt *et al.*, 1995). In high turbid water there are an increased number of suspended particles available for adsorption and colloidal charge neutralization. The net effect is an increase in particle collision frequency and formation of flocs which settle faster than the colloids alone (LaMer and Healy, 1963; Birkner and Morgan, 1968). Our experiments thus show that MO extract will be useful in reducing turbidity levels in high turbid waters which is also indicated by full scale treatment trials at a water treatment plant in Malawi where MO extract was used as the primary coagulant with promising results (Sutherland *et al.*, 1994). Results showed that inlet water turbidities during the trials ranged between 270 and 380 NTU and the final treated water turbidity was consistently below 4 NTU.

In contrast to the findings with high turbidity water, treatment of water with low turbidity (<50 NTU) in our study did not result in any significant reduction in turbidity, which is in accordance with other studies (Muyibi and Okufu, 1995; Dorea, 2006). The reason for this is that low turbid waters contain low concentrations of suspended particles and that MO proteins have a low molecular weight, resulting in smaller and lighter flocs (Bratby, 2006). We found increased

turbidity in tap water added MO which can be attributed to the fact that MO extract itself has some level of turbidity.

In this study, a settling time of 90-120 min was obtained with the optimum dosage of 100 ml of 3% w/v MO extract per litre in field experiments and at 4-8 ml of 5% w/v MO extract per litre in laboratory experiments. Reports on suspended solids of raw palm oil mill effluent treated with MO extract showed a settling time of 114 min (Bhatia *et al.*, 2007a; Bhatia *et al.*, 2007b). A study by Ndabigengesere *et al.* (1995) reported that more concentrated MO extract solutions are better since small volumes are required to achieve the optimum dosage. However, over-dosages can cause re-stabilization of the particles and consequently hinder the formation of flocs thus reducing sedimentation and hence removal of particles (Bhatia *et al.*, 2007b).

4.2 Removal of helminth eggs

Sengupta *et al.* (2011) showed that helminth eggs are denser than water and settle naturally with time and Keraita *et al.* (2008) showed that in wastewater sedimentation ponds there was a build-up of helminth eggs in the sediment with time. Our findings clearly show that in water not treated with MO extract the number of suspended helminth eggs decreased and that the number of eggs in the sediment increased with time, which supports the finding that helminth eggs settle naturally in water. However, treatment with MO extracts enhanced the settling of helminth eggs significantly. Reductions in helminth egg numbers by 94-99.5% was obtained in different water types of medium to high turbidity. Previous studies have shown that MO extract can reduce the number of fecal coliforms with up to 99.9% (Madsen *et al.*, 1987; Broin *et al.*, 2002; Ghebremichael, 2004) and the number of schistosome cercariae up to 90% (Olsen, 1987). *Ascaris* eggs have a small negative surface charge (Capizzi and Schwartzbrod, 2001; Dunn, 1991) which enables MO proteins to bind to egg surfaces through the same mechanism of action as explained above for particles (in section 4.1) resulting in increased coagulation and flocculation. This could explain why removal of *Ascaris* and possibly also *Trichuris* eggs is more efficient when adding MO extract to tap water with low turbidity. In water with medium or high turbidity, the helminth eggs probably also adhere to the particle flocs formed by coagulating proteins in MO extract since the surface of eggs are sticky (Gaspard *et al.*, 1994). The same mechanisms have also been speculated for coliforms and cercariae (Olsen, 1987; Pritchard *et al.*, 2010). The helminth eggs could also be captured by settling particle flocs with higher settling velocity than the eggs themselves, or maybe a combination of all these

processes. It has been shown by Sengupta *et al.* (2011) that helminth eggs flocculate with suspended particles in wastewater, and settle with the same velocity as the particles (*Trichuris*) or faster than the average particles (*Ascaris*).

With the settling time of 60-120 min an optimum reduction of helminth eggs was achieved with a MO extract solution concentration of 3% and 5%, for field and controlled experiments, respectively. The optimum dosage was determined based on reduction in turbidity which may not have been the optimal dosage for removal of helminth eggs. It should be noted that the initial number of suspended helminth eggs (app. 2000 eggs per litre) in laboratory experiments was more than a hundred times higher than the number of naturally occuring helminth eggs (14 eggs per litre) in the water used for the field experiments, however, still the efficiency of MO extract to remove helminth eggs was more or less the same. This is very promising since it indicates that MO extract is effective in removing helminth eggs at different concentrations which is typically the case for e.g. *Ascaris* in raw watsewater where 10-100 eggs per litre are often reported in endemic areas and 100-1000 eggs per litre are found in wastewater in hyperendemic areas (Mara and Sleigh, 2010; Kamizoulis, 2008).

4.3 Correlation between turbidity and helminth eggs

Regular monitoring of helminth eggs in irrigation water is expensive and requires skilled personnel. An important step in simplifying water quality measurements to allow for more regular monitoring is to establish a relationship between concentration of helminth eggs and a proxy parameter that is much easier and cheaper to measure, i.e. turbidity. In our study, a positive correlation was established between reduction of turbidity and helminth eggs in irrigation water, turbid water and wastewater treated with MO extract. This indicates that helminth eggs do attach to suspended particles and/or flocs facilitated by addition of MO extract to the water, and that helminth eggs and turbidity are reduced with the settling of flocs. This correlation between helminth egg numbers and turbidity is in agreement with a report by Pritchard *et al.* (2010) which indicated that reduction in numbers of *E. coli* was directly associated with reduction in turbidity following MO extract treatment. In our study, the correlation was strongest in the laboratory experiments were helminth eggs were added to the water at a concentration of approx. 2000 eggs per litre which is in contrast to the irrigation water in Ghana containing around 14 eggs per litre. It could be speculated that the high number of eggs in the laboratory experiments partly explains the relatively strong correlation

found. Additional studies of MO treated water with naturally occurring helminths and correlation analysis are needed to confirm our findings.

4.4 MO extract water treatment in the field

The optimum reduction of helminth eggs was generally higher with higher initial water turbidity. In turbid water and irrigation water, treatment with MO extract resulted in 1-2 helminth eggs per litre which is close to the WHO recommendations of ≤1 egg per litre in water to be safely used for irrigation (WHO, 2006). Whereas MO extract treatment of wastewater and tap water with lower turbidity yielded in helminth egg concentration much higher than the WHO guideline value. The reduction of turbidity to 7-11 NTU obtained in this study was close to the guideline value of ≤ 5 NTU by WHO (2006). Thus overall, MO extract was highly effective in reducing both helminth egg numbers and turbidity. For additional improvement of water quality it has been proposed to use sand filtration after MO extract treatment to obtain a further reduction of bacterial and parasite pathogens (Lea, 2010; Pritchard *et al.*, 2010). However, more studies are needed on how MO extract may affect crop productivity and the costs involved in employing MO extract treatment of large volumes of irrigation water. Development of simplified farmer-friendly MO extraction methods is needed as is further field trials to confirm the effectiveness of MO extracts to remove helminth eggs and other pathogens in different qualities of water.

5. Conclusion

Based on field and laboratory experiments it was found that MO extract was effective in reducing the number of helminth eggs by 94-99.5% and to reduce turbidity by 85-96% in different water types. Even though a relatively strong correlation was shown between turbidity and helminth eggs in turbid water and wastewater, it remains to be verified if turbidity can serve as a proxy for concentration of helminth eggs. MO is readily available in many tropical countries and can be used by farmers to treat high turbid water for irrigation, however, additional improvements of water quality, e.g. by sand filtration, is suggested to meet the guideline value of ≤ 1 helminth egg per litre and a turbidity of ≤ 5 NTU as recommended by World Health Organization.

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4.3.1 Additional results and discussion from Moringa-assisted sedimentation experiments (**Paper III**)

This section only comprises additional results from the water types used in the laboratory experiments in Denmark, i.e. turbid water, wastewater, and tap water. From Figure 4.3 it is seen that the suspended particles in both turbid water and wastewater are not equal in size but demonstrate a range of sizes.

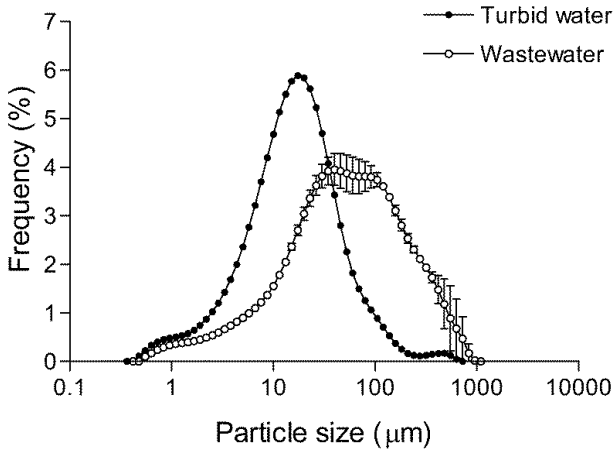

Fig 4.3. Particle size distribution in turbid water and wastewater used in laboratory experiments in Denmark before addition of *Moringa oleifera* extract. Note the log-scale on the x-axis.

Figure 4.4 shows suspended particles (turbidity) in turbid water and wastewater as a function of settling time both with and without addition of *Moringa oleifera* (MO) seed extract. Graphical inspection of the log-plot in Figure 4.4 reveals that the line connecting the data points (as described in section 3.4.4) is not straight. A log-plot of suspended helminth egg numbers as a function of settling time in turbid water, wastewater, and tap water both with and without addition of MO seed extract is seen in **Paper III** (Figure 3) and does not show an entirely straight connecting line either. The slope of the graphs is most steep from 0 min to 15 min where after it decreases. This indicates that not all particles or all helminth eggs settle with the same velocity, most likely because of a size distribution of both particles (as shown in Fig. 4.3) and eggs.

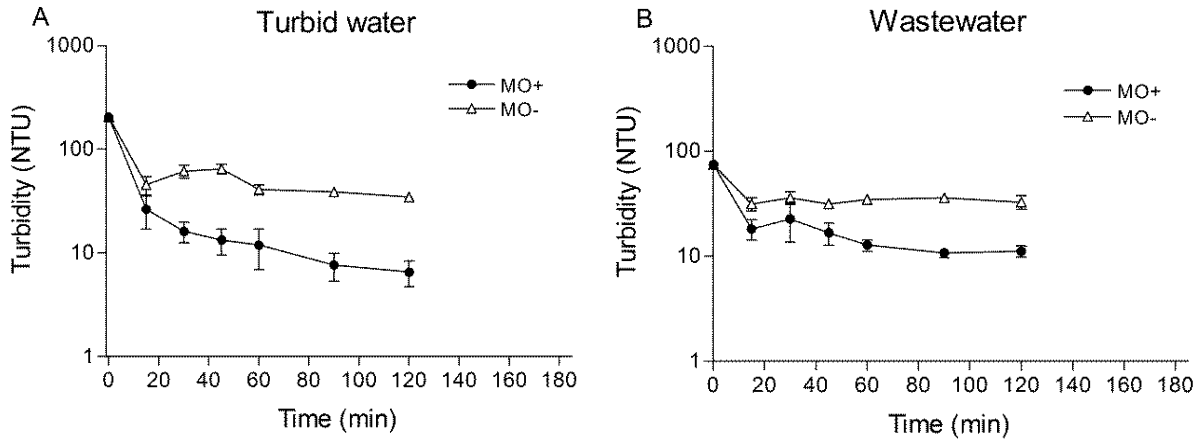

Fig. 4.4 Plot showing the relationship between turbidity and settling time in turbid water (A) and wastewater (B) with (MO+) and without (MO-) addition of *Moringa oleifera* extract.

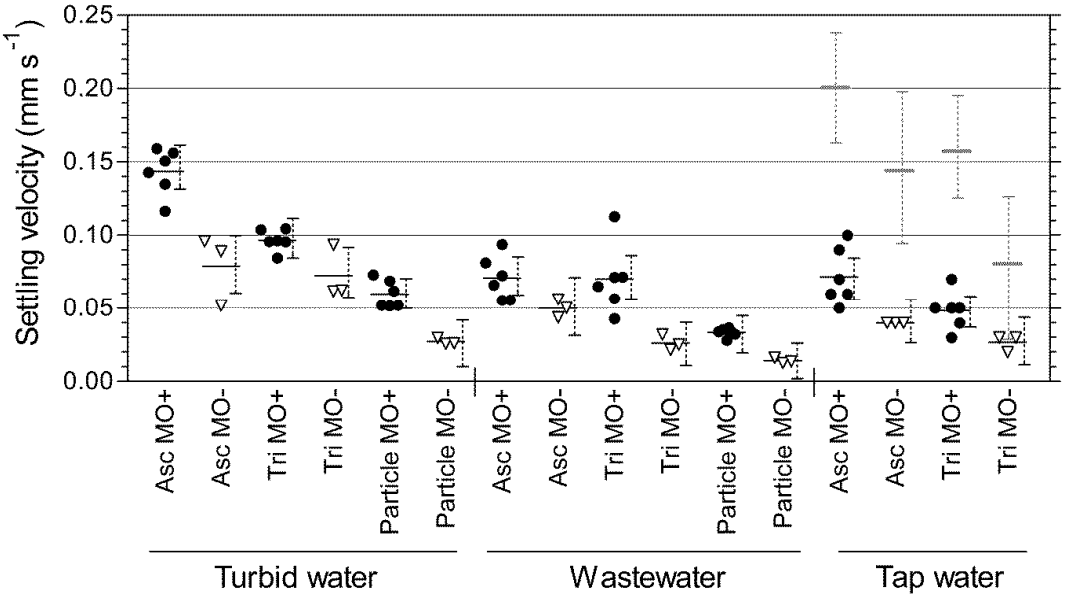

Fig. 4.5 Settling velocity within the first 60 min of settling (circles and triangles) of *Ascaris* (Asc) and *Trichuris* (Tri) eggs, and particles with (MO+) and without (MO-) addition of MO extract in three types of water i.e. turbid water, wastewater and tap water. Settling velocity within the first 15 min of settling (grey lines) of *Ascaris* (Asc) and *Trichuris* (Tri) in tap water. Mean±95%CI.

From 60 min to 120 min of settling the turbidity and number of suspended helminth eggs were relatively unchanged in all water types. Thus a mean settling velocity of particles and of each helminth egg types within the first hour was calculated and is shown in Figure 4.5. In the figure it is seen that in all three water types, i.e. turbid water, wastewater, and tap water, there is a tendency of enhanced settling velocity of both helminth eggs and particles with addition of MO seed extract. By multivariate ANOVA it is seen that addition of MO seed extract significantly increases the settling velocity in turbid water, wastewater, and tap water (Table 4.1). In turbid water the interaction between MO and particle type is also significant (P=0.017) and hence a post hoc test was performed for this case as well (Table 4.2). In turbid water, the settling velocity of *Ascaris* eggs and particles is

Table 4.1 P-values from the two-way ANOVA for each water type with settling time of 60 minutes or 15 minutes. NS: non-significant.

	Variable		
Water type	Particle type[a]	MO	Interaction
Turbid water (60 min)	<0.0001	<0.0001	0.017
Wastewater (60 min)	0.0001	<0.0001	NS
Tap water (60 min)	0.020	0.005	NS
Tap water (15 min)	0.030	0.005	-

[a]*Particle type is Ascaris eggs, Trichuris eggs, or particles*

enhanced by MO as compared to no addition of MO. MO-assisted settling of *Ascaris* eggs is significantly faster than both *Trichuris* eggs and particles, and *Trichuris* eggs settle faster than particles. With no addition of MO, *Ascaris* and *Trichuris* eggs settle at approximately the same velocity, but faster than the particles. In wastewater, the MO-assisted settling velocity is approximately the same for *Ascaris* and *Trichuris* eggs, but significantly faster than for the particles (P=0.002-0.003). With no addition of MO the settling velocity of *Ascaris* eggs, *Trichuris* eggs, and particles is not significantly different.

Table 4.2 P-values of the Tukey post hoc test of the significant interaction in turbid water. NS: non-significant.

	Asc MO-	Tri MO+	Tri MO-	Particle MO+	Particle MO-
Asc MO+	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Asc MO-	-	NS	NS	NS	0.002
Tri MO+	-	-	NS	0.002	<0.0001
Tri MO-	-	-	-	NS	0.007
Particle MO+	-	-	-	-	0.036

In tap water, the settling velocity of *Ascaris* and *Trichuris* eggs is overlapping both with and without MO seed extract, respectively. When calculating the mean settling velocity of helminth eggs in tap water within the first 15 min of settling (see Fig 4.5) there is also a significant increase in settling velocity with addition of MO (see Table 4.1). By comparing settling velocities of 15 minutes of settling with 60 minutes of settling, it is seen that the settling velocities are all significantly faster within the first 15 min of settling (P=0.002-0.009).

The settling velocity results demonstrate that addition of MO seed extract generally increase the settling velocity of helminth eggs and particles in different water types as compared to no addition. Even though the effect of MO on settling velocity was not statistically significant for all helminth eggs or particles in all three types of water the tendency of enhanced settling velocity was clear. This observation supports the results from **Paper III** showing that the use of MO seed extract can reduce both turbidity and helminth egg numbers in different types of water.

Chapter 5

Discussion of results and their implications

In this chapter the results from the three sub-studies presented in Chapter 4 and their implications will be discussed.

5.1 Settling velocity of different helminth egg types

Ascaris, *Trichuris*, and *Oesophagostomum* eggs represent the ascarid, the enoplid, and the strongyle type of eggs, respectively. From **Paper I** it is evident that eggs within each egg type are not uniform in size, but display a range of sizes (Fig. 5.1). Due to this size distribution the settling velocity too covers a range of values because larger eggs settle faster than smaller eggs. Likewise size and settling velocity distributions are also seen for non-flocculated primary particles, both in **Paper II** and in the literature (e.g. van Leussen, 1999). Thus, the settling velocity presented in **Paper I, II, and III** and reported in the literature is often the median settling velocity which is the velocity at which 50% of the particles or helminth eggs settle.

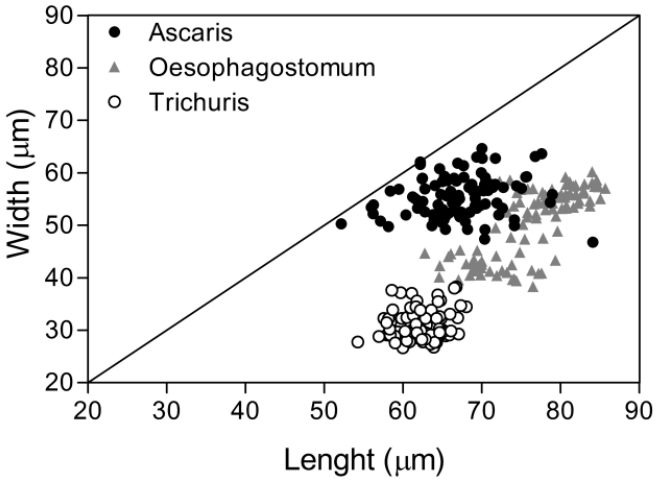

Fig 5.1 Egg size distribution of helminth eggs (from **Paper I**).

The settling velocity of helminth eggs in clean water was determined in all three sub-studies and findings are summarized in Table 5.1. Some variation in median settling velocities was seen which is most likely due to differences in size and/or density of the eggs rather than differences in the applied methods. The methods are fundamentally similar (sedimentation method) and therefore do not introduce systematic variance in the data (T.J. Andersen, personal communication). Assuming that settling of helminth eggs in water follows Stokes' law it is apparent that changes in egg size as compared to

density has a much larger effect on settling velocity since size (d) and density (ρ_p) parameters appear in 2nd and 1st order, respectively, i.e. $V_s = g/18 \times d^2 \times (\rho_p - \rho_l) \times \eta^{-1}$. This will also be the case for particles provided that Stokes' law is applicable (Droppo, 2001). The helminth eggs used in the three sub-studies were obtained from fresh faeces by the same sieving and flotation method, but the eggs originated from different worm populations which may explain the differences in size distribution of the three egg batches used. It has been documented that helminth eggs in the environment exhibit individual density variations depending on age or stage of development, e.g. degree of embryonation (Magat *et al.*, 1972; Sawitz, 1942). Even for the fresh helminth eggs used in **Paper I** some variation in egg densities was observed for each egg type. The difference in median settling velocities could also be a result egg aggregation in the water column despite of no particles being present.

Table 5.1 Overview of helminth egg settling velocities in tap water determined in the three sub-studies.

	Settling velocity, mm s^{-1} (95% CI)		
	Ascaris eggs	*Trichuris* eggs	*Oesophagostomum* eggs
Sedimentation exp. (paper I)			
Theoretical (Stokes's law)	0.285 (0.269-0.281)	0.129 (0.127-0.131)	0.158 (0.150-0.161)
Experimental	0.061 (0.057-0.066)	0.149 (0.096-0.202)	0.126 (0.107-0.145)
Resuspension exp. (paper II)			
Day 1	0.058 (0-0.181)	0.026 (0-0.171)	ND
Day 3	0.048 (0-0.137)	0.067 (0-0.209)	ND
***Moringa*-assisted sedimentation exp. (paper III)**			
First 60 min of settling	0.071 (0.059-0.083)	0.048 (0.036-0.061)	ND

ND: Not determined.

5.2 Enumeration of helminth eggs

In **Paper I** the settling velocity of three helminth egg types in clean water was determined experimentally and compared to values predicted by Stokes' law. The results showed that for *Trichuris* and *Oesophagostomum* eggs there was good agreement between the prediction by Stokes' law and the observed settling velocities whereas for *Ascaris* eggs the predicted settling velocity was overestimated compared to the observed value. As mentioned in Chapter 2, Stokes' law implicitly assumes that the settling particle has a spherical morphology with a smooth surface and that it is not subjected to aggregation. Thus, the overestimation of *Ascaris* settling velocity could be explained

by the fact that *Ascaris* eggs are mammilated and not smooth as is the surface of the other two helminth egg types (Fig. 5.2).

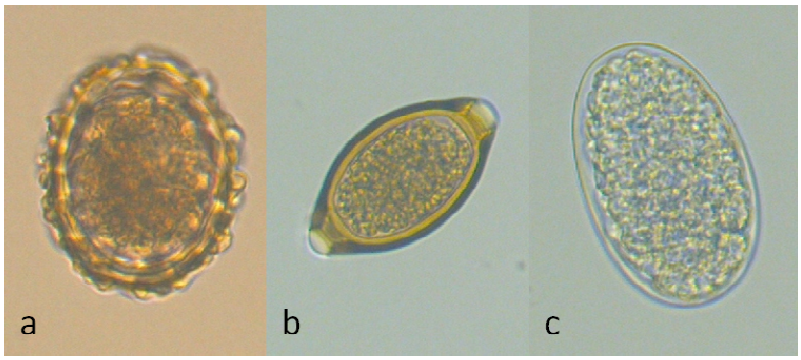

Fig. 5.2 Morphology of the three pig helminth eggs. Not to scale. Photos by the author. a. *Ascaris suum*; b. *Trichuris suis*; c. *Oesophagostomum* spp. (same as Fig. 2.1)

Many methods for quantification of helminth eggs in wastewater, sludge, and soil are described in the literature. A single method that can recover all helminth egg types with a known recovery rate is not available. Some methods are time-consuming while others use expensive or harmful chemicals. All published methods are based on either flotation, sedimentation, or a combination of the two. The WHO recommends using the modified Bailenger method (Ayres and Mara, 1996) for enumeration of helminth eggs in wastewater. In this method sedimentation time is based on calculation of helminth egg settling velocities using Stokes' law and the settling time is at least doubled to ensure collection of all eggs, i.e. 0.17 mm s^{-1} (*Ascaris*), 0.14 mm s^{-1} (*Trichuris*), and 0.05 mm s^{-1} (hookworm) (Ayres and Mara, 1996). By comparison with experimentally derived helminth egg settling velocities (**Paper I**), i.e. 0.06 mm s^{-1} (*Ascaris*), 0.15 mm s^{-1} (*Trichuris*), and 0.13 mm s^{-1} (*Oesophagostomum*), it is evident that *Trichuris* and *Oesophagostomum* eggs will settle within the sedimentation time recommended by the modified Bailenger method. However, the settling velocity of *Ascaris* eggs is overestimated by the modified Bailenger method (**Paper I**), thus the number of eggs in a wastewater sample would be underestimated.

In the USEPA modified concentration method (Schwartzbrod, 1998) used in **Paper III**, a sedimentation time of at least 3 hours is used. Prior to sedimentation the water sample is diluted with 5 litres of clean water corresponding to a settling height of 15.9 cm when using a plastic bucket with bottom diameter of 20 cm. From the experimental settling velocities (**Paper I**) it can be determined that after 3 hours of settling the helminth eggs would have settled 65 cm (*Ascaris*), 162 cm (*Trichuris*), and 140 cm (*Oesophagostomum*). Thus with a sedimentation time of 3 hours all helminth eggs in the 5-litre water sample would have settled. However, all theoretical calculations using Stokes' law are based on settling of particles in clean water whereas water samples to be

analyzed will contain different particles in different concentrations, e.g. wastewater and irrigation water.

In **Paper I** and **II** it was demonstrated that helminth eggs interact with suspended particles and get incorporated into particle flocs which then determine the settling behaviour of the eggs. Hence it is not appropriate to predict settling velocities using Stokes' law on helminth eggs assuming that the eggs settle in isolation since they clearly interact with suspended material. Aggregation of suspended helminth eggs and particles must be taken into account when predicting settling behaviour of the eggs as well as when determining sedimentation time of helminth eggs in the different enumeration methods, e.g. the modified Bailenger method. We found that flocculation typically enhances settling velocity of particles and eggs which implies that the current helminth egg enumeration methods, i.e. the modified Bailenger method and the USEPA modified method, in this case do not underestimate egg concentration. However we also found that flocculation can decrease the settling velocity of helminth eggs (**Paper I**) which indicates that whether the settling velocity is enhanced or decreased may depend on the particular particle composition (discussed in section 5.3).

In the present study only sedimentation of nematode eggs within the helminth 'order' was investigated (**Paper I, II,** and **III**). However the findings may be useful also when evaluating methods for enumeration of trematode eggs, which usually are based on sedimentation due to the high density of trematode eggs (Roepstorff and Nansen, 1998). In the FAO guidelines (Roepstorff and Nansen, 1998), developed for detection of e.g. *Fasciola hepatica* eggs in pig faeces, samples are mixed with water, sieved, and then allowed to settle twice for 10 min. In a special technique developed for detection of *Schistosoma japonicum* eggs in faeces samples the eggs are also sieved, but sedimentation time of 20 min and 15 min is recommended (Willingham *et al.*, 1998). Many of the trematode eggs are relatively large (larger than 50 µm) and with their high density these sedimentation times are probably sufficient for sedimentation if using e.g. 15 cm high conic sedimentation beakers. However, for small trematode eggs with a length less than 50 µm (Dung *et al.*, 2007) it could be speculated that the rather short sedimentation time of 10 to 20 min is not sufficient for them to settle and may result in underestimation of actual egg numbers. Even though the small trematode eggs have a high density as compared to helminth eggs (Harnnoi *et al.*, 1998) the smaller size might slow down sedimentation as discussed in section 5.1. For trematode eggs a

possible interaction with suspended particles should also be taken into account as for nematode eggs since it may influence the sedimentation time.

5.3 Sedimentation of flocs

Apart from flocculation in the water column, results from **Paper II** showed that erodibility of both sediment and helminth eggs decreased over time, indicating that consolidation of the sediment takes place and eggs are incorporated in the sediment, probably through adhesion of eggs to sediment particles and biological activity, e.g. of bacteria . This is further supported by the settling velocity experiments from **Paper II** where a much higher settling velocity was found for helminth eggs associated with particles as compared to eggs in clean tap water. Settling velocity of flocs can also be calculated by Stokes' law if floc size and effective density of the floc is known (Liss *et al.*, 1996). However, both of these parameters are difficult to measure since flocs can be delicate porous structures with high water content and thus not an impermeable spherical morphology as Stokes' law assumes. In a study by Johnson *et al.* (1996) the theoretical settling velocity of highly porous non-spherical aggregates was compared to the observed settling velocity. The observed settling velocity was found to be 4-8 times higher than predicted by Stokes' law due to increased water flow through pores in the aggregate.

Most studies have found the general relationship that as floc size increases, settling velocity increases (Kim and Stolzenbach, 2004; Droppo *et al.*, 2000). The results from **Paper II** support these findings since the settling velocity of aggregated cohesive particles were twice as high as would be calculated on the basis of the primary grain size distribution using Stoke´s law (0.73 mm s^{-1}). However, a very low settling velocity of flocculated wastewater particles were found in **Paper I** as compared to settling velocities reported in saline water indicating that the water content of the wastewater flocs was very high (Gibbs, 1983). According to the literature floc size seems to have a much stronger effect on floc settling than density (Droppo *et al.*, 2000). Even though the interaction between helminth eggs and particles in the water column and on the sediment bed has been established in **Paper I** and **II,** the settling behaviour of particle-egg flocs are not straight forward. The settling of flocs appears to be influenced by the concentration, composition, and organic/inorganic content of the floc as well as the ionic composition of the water, thus making it difficult to predict the settling behaviour of flocs, e.g. by Stokes' law.

5.4 Helminth egg mobility in flowing water

When constructing water channels, e.g. for irrigation purposes, it is important to control the water flow to deliver the correct amount of water to the irrigated fields as well as to limit erosion of the canal sediment and banks hindering downstream blocking by deposited sediment and collapse of banks (Brouwer *et al.*, 1985). Sediment cohesion and aggregation has a marked effect on both the erosion potential and settling velocity of the sediment, typically resulting in increased erosion resistance of sediment beds and increased settling velocity of suspended sediment (Andersen and Pejrup, 2002; Nowell *et al.*, 1981). Sediment particles deposited on fine-grained beds in nature are incorporated into the sediment bed over time due to the range of biological and physical-chemical processes (section 2.3). This is supported by the results from **Paper II** where the erosion potential of the cohesive sediment bed decreased over time. **Paper II** also showed that *Ascaris* and *Trichuris* eggs adhere to the fine-grained particles on the bottom and are eroded along with the sediment and subsequent flocculation in the water column further increases their aggregation. This adherence to and incorporation into the bed decrease the mobility and transport of the eggs, and aggregation in the water column increases the effective settling velocity of helminth eggs which also results in reduced mobility.

Fig. 5.3 Irrigation channel in Malawi. Photo: FISD/IPS www.ips.org/africa

In **Paper II**, the maximum erosion threshold of about 0.1 N m^{-2} for sediment and 0.05 N m^{-2} for helminth eggs was demonstrated. To be able to use these results when constructing water channels (Fig. 5.3) the erosion threshold was recalculated to a flow velocity where the sediment and eggs begin to erode. The flow velocity recalculation has been done for an open irrigation channel in bare soils without vegetation (Engelund and Pedersen, 1978) and a water depth of 1 m. The calculated critical mean flow velocities are 0.10-0.18 m s^{-1} for the sediment and 0.07-0.12 m s^{-1} for the helminth eggs. These velocities are well below typical flow speeds for irrigation channels (van den Bosh *et al.*, 1992), and helminth eggs will consequently show high mobility if

introduced into typical irrigation channels. Hence removal of eggs from irrigation water by sedimentation will therefore only take place in rather stagnant water i.e. lakes or wastewater stabilization ponds. Entrapment of eggs by vegetation in the water stream or channel will occur and egg numbers will also decrease when eggs are eaten by fish or other macro-fauna on the sediment bed. However due to the experimental setup in **Paper II** where helminth eggs were added after sediment bed preparation the results are only valid for newly deposited eggs. If longer time had been applied for sediment and helminth egg consolidation, the erosion potential of both sediment and eggs would most likely have decreased. In natural aquatic environments both physical and biological properties of sediments may display seasonal variation, and hence temporally varying erosion potential and settling velocity (Andersen, 2001).

5.5 Enhanced sedimentation of helminth eggs by *Moringa oleifera* seed extract

In natural waters the particle concentration (turbidity) varies much as does the particle sizes, and this will have significant impact on the flocculation and sedimentation processes of such particles. As mentioned in Chapter 2 (section 2.2) fine particles suspended in freshwater stay dispersed because of the dominance of electrostatic repulsion over the attracting Van der Waals force (Russel *et al.*, 1989). **Paper I** and **II** demonstrated that helminth eggs interact with suspended particles in water. Thus if the particles stay suspended it would be expected that some of the helminth eggs would too. Water to be used for irrigation of edible crops should contain less than one egg per litre as recommended by WHO, thus water treatment is needed to enhance the sedimentation of helminth eggs if contaminated water is to be used for crop irrigation, e.g. by use of a natural coagulant like *Moringa oleifera* (MO) seed extract (**Paper III**).

The settling velocity results presented in Fig 4.5 demonstrate that addition of MO seed extract generally increased the settling velocity of helminth eggs and particles in different water types**.** Even though the effect of MO on settling velocity was not significant for all helminth eggs or particles in all three types of water the tendency of enhanced settling velocity is clear. In **Paper III** the effect of MO seed extract was significant and reductions were obtained in both helminth egg numbers by 94-99.5% and turbidity by 85-96% in different water types of medium to high turbidity. In **Paper III** the helminth egg reduction corresponds to a remaining egg concentration of 1-2 eggs per litre which is close to the WHO recommendations for water to be used for irrigation with no

restrictions. If this treatment method is to be implemented on farmer level the use of MO seed extracts to treat large volumes of water must be cost effective. In Ghana one MO seed of 3-4 g costs about US$ 0.6 and 3 kg of seed are needed to treat 30,000 litres of water (Doerr, 2005). A single MO tree produces 50-70 kg of pods with each pod containing approximately 10 seeds and the cost of producing 1 kg MO seeds (3,400 seeds) is about US$ 2 (Goh, 2005). The active coagulant proteins can be extracted either from the grounded MO seeds, where after the remaining cake residue can be used as animal fodder or plant fertilizer, or from the solid residue left after extraction of oil from the seeds (Muyibi *et al.*, 2002). Based on the multiple purpose use of the different parts of the MO seeds and the low price, use of MO seed extract to treat low quality water seem to be applicable at farmer level. Apart from the mentioned costs a land area is required for the sedimentation pond (Fig. 5.4) and also both costs and time spent on protein extraction, digging, and maintenance of a sedimentation pond must be taken into account. It has also been proposed to use sand filtration after treatment with MO seed extract for obtaining further reduction of bacterial and parasite pathogens to meet the WHO guidelines (Pritchard *et al.*, 2010; Lea, 2010). Additionally, development of simplified farmer-friendly MO extraction methods is required. The optimum reduction of helminth eggs was generally higher with higher initial water turbidity (**Paper III**) so determination of water turbidity prior to treatment with MO seed extract is important. Overall, on-farm field trials are needed for evaluating MO seed extracts as a possible low technology water treatment method.

Fig. 5.4 Peri-urban farmers collecting water from sedimentation pond for irrigation in West Africa (Photo: International Water Managemenr Institute).

5.6 Surface properties of helminth eggs

Extraction of helminth eggs from e.g. faecal samples is not just a matter of sedimentation and flotation based on density differences between the eggs and the extraction medium according to Bailenger (1979). Extraction of helminth eggs also depend on their hydrophilic surface properties which can be modified by pH. Bailenger (1979)used centrifugation of a diphase water-ether emulsion (ether above, water below) for extraction and showed that the hydrophilic-lipophilic

balance of *Ascaris* spp. and hookworm eggs can be modified by pH. The higher pH (from 4 to 10) the greater number of *Ascaris* eggs were extracted whereas the optimal pH zone for hookworm egg extraction was 4 to 7. The pH did not seem to affect the extraction of *Trichuris* eggs. Bailenger (1979) speculates that helminth egg surfaces (Fig. 5.5) are hydrophilic whereas faecal particle surfaces are lipophilic so by changing the pH the undefined surface molecules on both eggs and particles change. This leads to a change in the hydrophilic-lipophilic balance and hence variations in the egg recovery. In the modified Bailenger method (Ayres and Mara, 1996) recommended by WHO, extraction is done at pH 4.5 which may not be the best for all eggs, but it is a trade-off between recovery of eggs and recovery of too many particles hindering the enumeration of eggs. A few other studies have been carried out to describe surface properties of *Ascaris* eggs. Gaspard *et al.* (1994) demonstrated that *A. suum* eggs had some affinity for hydrophilic surfaces, such as glass. However in a study by Capizzi and Schwartzbrod (2001) some affinity of *A. suum* for lipids with hydrophobic properties was shown. These two studies applied different methods for testing the hydrophobic-hydrophilic affinity of *A. suum* eggs, thus the results of the eggs having both hydrophilic and hydrophobic surface properties are plausible when it is kept in mind that pH value, ionic composition of the medium, and temperature affect the egg surface properties (Gaspard *et al.*, 1994). *Cryptosporidium parvum* oocysts has been shown to have a low hydrophobic affinity, indicating a preferred adhesion of oocysts to glass surfaces as opposed to hydrophobic plastic materials (Drozd and Schwartzbrod, 1996). The same study showed that the negative charge of oocyst surfaces reached 0 at pH 2.5, thus making adhesion to particles possible due to the elimination of electrostatic repulsion between oocysts and particles. For bacteria it has been shown that elevated ionic strength in wastewater decreased the surface charge of bacteria (Grotenhuis *et al.*, 1992), thereby also enabling adhesion to particles. In **Paper I, II, and III** it was demonstrated that helminth eggs adhere to particles and become incorporated in particle flocs. However, it is still

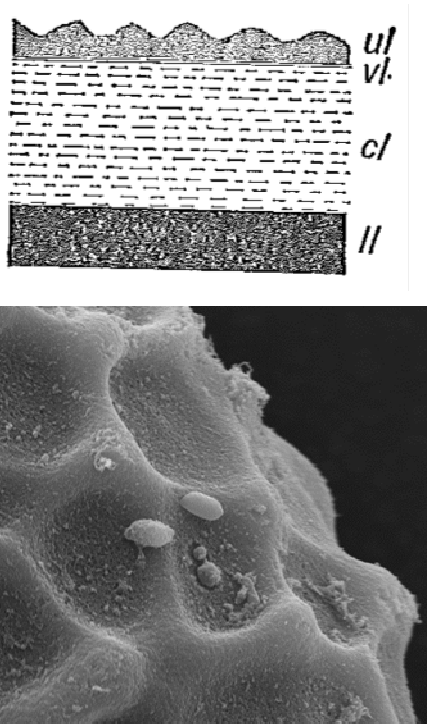

Fig 5.5 Above: the egg shell structure of *A. lumbricoides* (same as Fig. 2.2).Layers: *ul* – uterine, *vl* –vitelline, *cl* – chitinous, and *ll* – lipid layer. Below: Scanning Electron Microscopy photo of *A. suum* shell surface (same as Fig 2.4).

unclear to which extent hydrophilic-hydrophobic surface properties of helminth eggs are involved in adhesion to particles and which other physical-chemical mechanisms are responsible for this adhesion.

5.7 Modelling the fate of helminth eggs

Knowledge of helminth egg behaviour in water can be used to develop models predicting the fate of eggs in natural settings, e.g. in river systems, to assess the water quality if the water is used for crop irrigation, recreational activities, or other purposes involving human exposure. A river model like the MIKE model developed by DHI Water, Environment, and Health in Denmark (www.mikebydhi.com) consists of a part describing the hydrology and a part describing the sediment. Information on the upstream setting of the river is required since it is essential to know what drives the water flow in the river. For example, in a water quality survey in the Musi-River in India the downstream river water from Hyderabad City was examined (Ensink *et al.*, 2010). In this study the upstream setting was the city which disposed large amounts of untreated wastewater into the river. Additionally, identification and quantity information on the sources contributing water and sediment into the river is needed, i.e. rainfall, sewage channels, smaller rivers, etc. Details on the sediment are also important in the model, i.e. composition (cohesive, sand, etc.), concentration, and particle size distribution, as these relate to transport and resuspension of the sediment. In the MIKE model properties can be added to the particles and helminth eggs can be viewed as particles (as mentioned in section 2.2). Hence information on helminth egg settling velocity and resuspension rates (**Paper I** and **II**) as well as egg viability (outside the scope of this thesis) can be added into the model. Data on sources contributing helminth eggs into the river are needed, e.g. a riverside village population with high helminth prevalence. From **Paper I** and **II** it is known that helminth eggs in water interact with particles both in the water column and on the sediment bed and hence this interaction may be a key point in the model. Since the contribution of both water and sediment from the identified sources changes during the year a seasonal variation must also be taken into account. Moreover temperature, light, and hence biological activity is known to be important in incorporation of helminth eggs into the sediment and the following sediment consolidation (Black *et al.*, 2002); **Paper II**) and will probably also influence the viability of the eggs. Whether the data presented in **Paper I** and **II** are sufficient and solid enough for developing a model predicting the fate of helminth eggs in natural settings is not known, but follow-up investigations are planned to

use the data generated in this PhD project to further develop existing water models, e.g. the MIKE model.

5.8 Study limitations

In this section limitations of the study designs and methodologies used in the three sub-studies presented in **Paper I, II,** and **III** will be discussed.

Even though the Owen tube method (**Paper I**) is widely used for directly measuring settling velocities of different types of sediment in freshwater, estuarine, and marine environments, and is a well-documented method, it still has some drawbacks. Inside the Owen tube some adhesion of particles and possibly also helminth eggs to the tube wall takes place and may result in contamination of one subsample by the previous one. When collecting subsamples the water level decreases, resulting in the last subsample having an increased concentration of both particles and helminth eggs (Mantovanelli and Ridd, 2006). This would not be the case in natural water. To address this problem it is assumed that the last subsample of water has the same concentration of particles and helminth eggs as the previous subsample. The concentration difference between these two subsamples is then added to all earlier subsamples relatively to their settling time.

In **Paper I** the diameter of the Owen tube (5 cm) could also be a problem since particle settling velocities are reduced in tubes with small diameters (Lovell and Rose, 1991). The fluid pulled along by settling particle produces a return flow due to the tube wall since it cannot pass through the wall (Dearnaley, 1996). However, according to Huisman (1995) the small diameter of the Owen tube is not a problem since the upward flow of displaced fluid does not significantly hinder the sedimentation if the particle has a size less than app. 1/45 of the column diameter. Hence in **Paper I**, sedimentation of particles of up to 1,100 μm in diameter can be determined. Helminth eggs are much smaller (mean size of 42-75 μm) and only a very small fraction of the wastewater particles were larger than 250 μm.

The number of added eggs in the resuspension experiment (**Paper II**) was approximately 12,000 eggs per litre which is a very high concentration of eggs as compared to helminth egg concentrations of natural wastewater or faecally contaminated water, e.g. between 10-2,000 eggs per litre (e.g. Bhaskaran *et al.*, 1956). Due to the experimental design where water subsamples of only 10 ml were collected for helminth egg quantification, a high concentration of eggs was

necessary to increase the sensitivity of the sampling to allow for subsequent statistical analyses of both erosion rates and settling velocities. In the WHO guidelines for wastewater analysis (Ayres and Mara, 1996) 10 litres of wastewater is recommended for sampling to ensure that low egg concentrations are detected.

The settling velocity part of the resuspension experiment (**Paper II**) was initiated when the propeller, inducing bed shear stress, was turned off. A settling time of six minutes was applied and during this time optical back-scattering (OBS) sensor readings were done every 10 seconds and subsamples for helminth egg quantification was taken every one minute. For calibration of the OBS readings subsamples were also taken for manual determination of the suspended sediment concentration. However, during some of the experiments the OBS reader was not working properly and hence the readings were discarded from the data set, and the determination of suspended sediment concentration by dry weight was used instead. This, however, was only done at time 0 min and 6 min, and therefore only two data points were available for calculation of settling velocity (as described in section 3.3.2).

In **Paper III** a two-way ANOVA analysis was performed on the number of helminth eggs and the turbidity in the water with addition of *Moringa oleifera* (MO) seed extract and time as fixed effects. Two levels of MO addition was applied, i.e. with (MO+) and without (MO-) addition. This might be a simplification due to the variation in the dosages used for each water type. However, determination of the optimum dosage with regard to turbidity was carried out for each of the water types used, i.e. irrigation water, turbid water, and wastewater. Optimum dosages of 100 ml of 3% w/v MO extract (irrigation water), 8 ml of 5% w/v MO extract (turbid water), and 4 ml of 5% w/v MO extract (wastewater) were obtained. These w/v dosages correspond to an actual addition of 35 g per litre, 0.4 g per litre, and 0.2 g per litre, respectively. The reason behind this variation in optimal dosages for the water types in Ghana and Denmark is unclear. In the field-based studies in Ghana volumes of 3% and 5% w/v MO seed extract were used for determination of the optimum dosage whereas only volumes of 5% w/v were tested in the laboratory-based experiments in Denmark. Even though the MO seed batches used in Ghana and in Denmark both originated from Kumasi in Ghana the quality and age may have been different.

Chapter 6

Conclusions and future studies

In this chapter the main conclusions from **Paper I, II,** and **III** are presented and it is discussed to what extent the objectives of the PhD project (section 1.1) have been achieved. In addition, a number of possible future studies derived from this PhD project are briefly described.

6.1 Conclusions

- Not all helminth (*Ascaris*, *Trichuris*, and *Oesophagostomum*) eggs in clean water settle according to Stokes' law which sometimes overestimates the settling velocity probably due to differences in surface morphology. This should be taken into consideration when Stokes' law is used to predict helminth egg removal in water, e.g. when designing water treatment facilities.
- In water containing particles the settling velocity of *Trichuris* and *Oesophagostomum* eggs is often similar to that of the particles in the water. *Ascaris* eggs often show an increased settling velocity regardless of which particles they are associated with.
- *Ascaris* and *Trichuris* eggs adhere to the fine-grained particles on the sediment bed and are eroded along with the sediment, and subsequent flocculation in the water column further increases their aggregation and results in decreased mobility.
- Helminth eggs should not be viewed as single entities in water systems when modelling and predicting the distribution and fate of eggs since both their erodibility and settling velocity are determined primarily by the mobility of the sediment present in the water.
- The erosion threshold of *Ascaris* and *Trichuris* eggs shows that even in relatively slow moving water the eggs will demonstrate high mobility.
- *Moringa oleifera* seed extracts can be used to remove helminth eggs from faecally contaminated and turbid water, but additional improvements of water quality is suggested to fully meet the guideline value of ≤ 1 helminth egg per litre as recommended by the World Health Organization for water used for crop irrigation.

The overall objective of increasing our understanding and knowledge of how helminth eggs behave in different types of water are fulfilled, but whether the specific data acquired in the PhD project is solid enough for modelling the fate of helminth eggs in water remains to be investigated. Additionally, more information on exactly how helminth eggs are incorporated into particle flocs is needed since this may depend on the type of particle. The specific objective of determining settling velocity and resuspension rate of different helminth eggs in water and sediment is met. However from the results obtained it is apparent that many factors such as water type, particle composition, and particle concentration affect settling and resuspension of helminth eggs, so although many of the main results are likely to represent a general behaviour, the specific and quantitative observations may be valid only for the water types studied in the PhD project. Hence further studies on egg surface properties and flocculation behaviour are suggested. The last specific objective of investigating to what extent seed extract of *Moringa oleifera* (MO) can be used as a coagulant to increase the settling velocity of helminth eggs is partly met. Some questions still remain to be answered regarding differences in optimal MO seed extract dosages between laboratory and field trials.

6.2 Future studies

- To test the hypothesis stating that the mammilated surface structure of *Ascaris* eggs is related to the overestimation of settling velocities predicted by Stokes' law, sedimentation experiments using Owen tubes should be carried out with *Ascaris* eggs where this outer mammilated protein layer has been removed, e.g. by NaClO treatment (Johnson *et al.*, 1998). If the mammilated layer of *Ascaris* eggs is responsible for turbulent flow around the egg it would be expected that the settling velocity of smooth *Ascaris* eggs would be in better agreement with predictions made by Stokes' law.
- To increase our knowledge on how helminth eggs are incorporated into particle flocs it is suggested to create particle flocs with different composition and entrapped helminth eggs. The morphological structure of these flocs and the way helminth eggs are entrapped in them can be investigated by video filming how such flocs develop and settle, or by multiple microscopy techniques (Droppo, 2001). By using conventional optical microscopy it is possible to obtain non-destructive observations and measurements of the flocculated material within a plankton chamber. An environmental scanning electron microscope can be used for observation of

hydrated flocs. Lastly, for more detailed structural observations transmission electron microscopy may be applied.

- The resuspension experiment carried out in this PhD project investigated the erosion potential of newly deposited helminth eggs. To generate information on helminth eggs which have been in the sediment for longer time a resuspension experiment should be carried out where the helminth eggs are mixed with the sediment prior to sediment bed preparation. It could be done by adding helminth eggs and sediment when the propeller (see experiment setup in Fig 3.4) turns at the lowest speed and then letting the sediment and eggs settle. A consolidation time longer than 1-3 days should be applied before performing the resuspension experiment. It is expected that helminth eggs well incorporated into the sediment would have lower erosion potential than newly deposited eggs.
- In the studies of Capizzi and Schwartzbrod (2001) and Gaspard *et al.* (1994) investigating *Ascaris suum* egg surface properties (mentioned in section 5.6) the eggs used for experiments were derived from worm uteri and the surfaces of these eggs are likely to be quite different from the tanned surfaces of *Ascaris* eggs collected from faeces (see section 2.1.3). Thus there is a need to determine the surface properties of helminth eggs collected from faeces. A possible experiment could be a 'Microbial Adhesion To Solvents' (MATS) assay where the affinity of *Ascaris* eggs for different solvents, e.g. hydrophilic or hydrophobic, could be explored (e.g. (Capizzi and Schwartzbrod, 2001; Drozd and Schwartzbrod, 1996). The information regarding affinity is useful for improving the purification of faeces-derived helminth eggs for enumeration and treatment of wastewater and sludge. Additionally studies on surface charges of different helminth eggs by electrophoresis could generate information relevant for understanding flocculation processes.

Chapter 7

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